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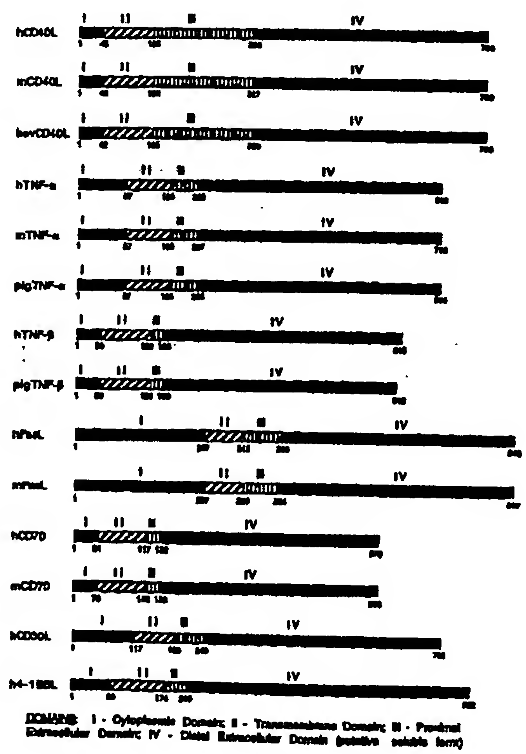
(57) Abstract

This invention relates to genes which encode accessory molecule ligands and their use for immunomodulation, vaccination and treatments of various human diseases, including malignancies and autoimmune diseases. This invention also describes the use of accessory molecule ligands which are made up of various domains and subdomain portions of molecules derived from the tumor necrosis factor family. The chimeric molecules of this invention contain unique properties which lead to the stabilization of their activities and thus greater usefulness in the treatment of diseases. Vectors for expressing genes which encode the molecules of this invention are also discussed.



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DESCRIPTION

Novel Expression Vectors Containing Accessory Molecule Ligand Genes And Their Use For Immunomodulation And Treatment Of Malignancies And Autoimmune Disease

Related Application

This application claims priority to Kipps et al., NOVEL EXPRESSION VECTORS CONTAINING ACCESSORY MOLECULE LIGAND GENES AND THEIR USE FOR IMMUNOMODULATION AND TREATMENT OF MALIGNANCIES, United States Provisional Application No. 60/132145, filed December 9, 1996, which is incorporated herein by reference including drawings.

Technical Field of the Invention

The present invention relates to novel expression vectors containing genes which encode an accessory molecule ligand and the use of those vectors for immunomodulation, improved vaccination protocols and the treatment of malignancies and autoimmune diseases. More particularly, this invention provides expression vectors and methods for treating various neoplastic or malignant cells, and expression vectors and methods for treating autoimmune Disease. This invention also contemplates the production and expression of accessory molecule ligands with greater stability and enhanced function.

Background of the Invention

Leukemias, lymphomas, carcinomas and other malignancies are well known and described in, e.g., Harrison's Principles of Internal Medicine, Wilson et al., eds., McGraw-Hill, New York, pp. 1599-1612. These malignancies appear to have somehow escaped the immune system surveillance mechanisms that eliminate rapidly and continuously proliferating cells. The exact mechanism by which these malignancies escape the immune system surveillance is not known.

Some of these malignant immune system cells are malignant antigen presenting cells which do not function properly within the immune cascade. For example, neoplastic B cells cannot induce even weak allogeneic or autologous mixed lymphocyte reactions in vitro. Further evidence that malignancies survive due to the failure of the immune surveillance mechanism includes the increased frequency of such malignancies in immunocompromised individuals, such as allograft recipients and those receiving long-term immunosuppressant therapy. Further, the frequency of these malignancies is increased in patients having Acquired Immune Deficiency Syndrome (AIDS) and patients with primary immune deficiency syndromes, such as X-linked lymphoproliferative syndrome or Wiscott-Aldrich Syndrome (Thomas et al., Adv. Cancer Res. 57:329, 1991).

The immune system normally functions to eliminate malignant cells by recognizing the malignant cells as foreign cells and clearing those cells from the body. An immune reaction depends on both the immune system's antibody response and on the cellular immune response within a patient. More specifically, the cellular immune response which acts to recognize the malignant cells as foreign requires a number of different cells of the immune system and the interaction between those cells. An immune reaction begins with a T lymphocyte (T cell) which has on its cell surface the T cell receptor. The T cell also has the ability to express on its surface various accessory molecules which interact with accessory molecules on the B lymphocyte (B cell). When the T cell receptor of the T cell specifically binds to a foreign antigen, such as a malignant cell, it becomes activated and expresses the accessory molecule ligand, CD40 ligand on its cell surface. The accessory cell molecule ligand is only present on the activated T cells for a short period of time and is rapidly removed from the cell surface. After the accessory cells molecule

ligand is removed from the surface of the activated T cell, its ability to bind to B cells via the accessory molecule ligand is destroyed.

When present on the surface of an activated T cell, the accessory cell ligand can specifically bind to the accessory cell molecule present on the B cell. This specific T-B cell interaction causes the B and T cell to express costimulutory surface accessory molecule and cytokines which result in an immune activation which lead to cytolytic T cells which specifically kill and remove the malignant cell from the body.

The interaction with an activated T cell is not solely limited to B cells but rather can be carried out by any cell which is able to present antigen to the T cell (an antigen presenting cell). These cells include B lymphocyte, macrophages, dendritic cells, monocytes, Langerhans cells, interdigitating cells, follicular dendritic cells or Kupffer cells. These cells all are known to have various accessory molecules on the cell surface which allow them to interact with other cells of the immune system. For example, these antigen presenting cells all have the accessory molecule CD40 on their cell surface. The presence of these accessory molecules allows these antigen presenting cells to specifically bind to complimentary accessory molecule ligand and thus directly interact with other immune cells.

A large number of accessory molecule ligands are members of the tumor necrosis factor superfamily. (Fanslow et al., Sem. Immun., 6:267-268 (1994). The genes for a number of these accessory molecule ligands have been cloned and identified. These accessory molecule ligand genes encode accessory molecules which all have the configuration of Type II membrane proteins and exhibit varying degrees of homology with other accessory molecule ligand genes. For example, the accessory molecule ligand genes encoding both murine

CD40 ligand and human CD40 ligand have been isolated. See, Armitage et al., Nature, 357:80-82 (1992) and Hollenbaugh et al., EMBO J., 11:4313-4321 (1992).

CD40 and its ligand, CD40 ligand are critical components of a normal immune response. CD40 mediated signals induce immune lymphocytes to proliferate and differentiate and become potent antigen presenting cells. Malignant or neoplastic B cells are poor antigen presenter cells and are unable to stimulate a vigorous allogeneic mixed lymphocyte reaction. Successful cross linking of CD40 molecules on immune cells results in a strong allogeneic mixed lymphocyte reaction suggesting a strong immune reaction. Various soluble CD40 ligands or antibodies specific for CD40 have been used to potentially cross link CD40. These soluble CD40 ligands and CD40-specific antibodies are not optimal for cross linking the CD40 molecules on antigen presenting cells and do not work as effectively as CD40 ligand expressed on a cell membrane to produce strong stimulation of antigen presenting cells. These methods are also difficult to implement because large amounts of CD40 ligand constructs or antibodies must be isolated which is difficult and time-consuming work. Other strategies to utilize CD40 ligand in solution or as a membrane bound molecule including transformation of fibroblasts with CD40 ligand to produce cultured cells which are then used to present antigen are not amenable to in vivo human clinical protocols.

CD95 (Fas) interaction with its ligand (Fas-ligand, or FasL) functions to limit the duration of the immune response and/or life-span of activated lymphocytes. Apoptosis induced by Fas-FasL binding serves to clear activated self-reactive lymphocytes. Problems caused by altering this pathway have been demonstrated in animals with defects in Fas<->Fas-ligand interactions. Mice having mutations, which inactivate CD95 or FasL, develop numerous disorders including autoimmune pathology

resembling that seen in patients with rheumatoid arthritis (RA) or systemic lupus. Zhang, et al., in J. Clin. Invest. 100:1951-1957 (1997) show that injection of FasL-expressing virus, into the joints of mice with collagen-induced-arthritis, results in apoptosis of synovial cells and relief of arthritis symptoms. Expression of Fas ligand allows clearance of activated cells which play a role in the pathogenesis of autoimmune disease. Therefore, a gene therapy strategy for introducing FasL into the joints of rheumatoid arthritis patients could function to improve disease pathology by leading to destruction of the infiltrating mononuclear cells.

Administration of soluble accessory molecules and accessory molecule ligands has been shown to trigger or to be associated with adverse physiological effects. For example, treatment of mice, having wild-type CD40-receptor expression, with soluble CD40L-CD8 fusion protein resulted in a pulmonary inflammatory response. This was not observed in mice in which the gene for the CD40 receptor had been knocked out. These experiments, described in Wiley, J.A. et al., Journal of Immunology 158:2932-2938 (1997), support *in vitro* data which suggest that CD40 ligation can result in inflammatory responses.

Direct administration of purified recombinant soluble Tumor Necrosis Factor (either α or β) results in shock and tissue injury, as described in Tracey, K. J., and A. Cerami, Annu. Rev. Med. 45:491-503 (1994). Within minutes after acute intravenous or intra-arterial administration of TNF, a syndrome of shock, tissue injury, capillary leakage syndrome, hypoxia, pulmonary edema, and multiple organ failure associated with a high mortality ensues. Chronic low dose of TNF causes anorexia, weight loss, dehydration and depletion of whole-body protein and lipid.

Soluble Fas ligand and receptor have also been shown to be associated with tissue damage and other adverse effects. CD95, the Fas receptor, is a mediator of apoptosis. Fas ligand induces apoptosis by binding to Fas receptor. As shown in Galle, P.R., et al., J. Exp. Med. 182:1223-1230 (1995) administering an agonistic anti-Fas antibody resulted in liver damage to mice. Mice injected intraperitoneally with the agonistic antibody died within several hours, and analyses revealed that severe liver damage by apoptosis was the most likely cause of death.

The role of soluble Fas ligand (FasL), in the pathogenesis of systemic tissue injury in aggressive lymphoma is described in Sato, K. et al., British Journal of Haematology, 94:379-382 (1996). The findings presented in this report indicate that soluble FasL is directly associated with the pathogenesis of liver injury and pancytopenia.

CD27, the receptor for the accessory molecule ligand, CD70, was shown, in a report written by van Oers, et al., in Blood 82:3430-3436 (1993), to be associated with B cell malignancies.

The above findings all contraindicate the administration of soluble accessory molecule ligands, highlighting the need for therapies that increase the levels of these molecules without resulting in an elevation of their soluble forms.

Despite the wealth of information regarding accessory molecule ligand genes and their expression on the surface of various immune cells, the exact mechanism by which the accessory molecule ligand genes are regulated on antigen presenting cells is not yet known. Without specific knowledge of the regulation of expression of accessory molecule ligand genes on these antigen presenting cells, altering the immune response by varying expression of an accessory molecule ligand gene has to date not been possible. Without any

specific knowledge as to how to regulate the expression of an accessory molecule ligand gene on an antigen presenting cell, it is not possible to alter the immune response towards malignant cells. Thus, there was a need for a method of increasing the expression of an accessory molecule ligand gene on normal and malignant cells including antigen presenting cells.

Further, without the ability to regulate the expression of accessory molecule ligands, it is not possible to alter the immune clearance of these cells.

Summary of the Invention

The present invention fills these needs by providing novel expression vectors containing accessory molecule ligand genes and methods for introducing those genes into normal and malignant antigen presenting cells thereby allowing the alteration of an immune response, the treatment of autoimmune diseases and the treatment of various neoplasias. This invention provides vectors, including gene therapy vectors which contain accessory molecule ligand genes. These vectors also contain the additional genetic elements, such as promoters, enhancers, polyadenylation signals (3′ ends), which allow that vector to be successfully placed within the cell and to direct the expression of the accessory molecule ligand gene in a cell. Such gene therapy vectors are capable of transforming animal cells directly and thereby introducing the accessory molecule ligand gene into the cells of that animal in a form which can be utilized to produce accessory molecule ligands within that cell.

In other aspects of the present invention, the function of an accessory molecule ligand is modified by altering the half life of the molecule on the cell surface or by changing the level of expression of that molecule on the cell surface. In preferred embodiments, the present invention provides accessory molecule

ligands which are modified to improve the stability of such accessory molecule ligands on the cell surface. Such increased stability may be accomplished using any of the disclosed methods of molecules described in this application, including chimeric molecules and molecules into which mutations have been introduced at least one location. The present invention also contemplates increasing the expression of such a molecule.

The present invention also provides gene therapy vectors containing the accessory molecule ligand genes which are chimeric in that portions of the gene are derived from two separate accessory molecule ligands which may or may not be from different species. The accessory molecule ligand genes of the present invention include genes which encode molecules of the tumor necrosis factor (TNF) family. The molecules which make up the TNF family include TNF_{α}, TNF_{β}, CD40 ligand, Fas ligand, CD70, CD30 ligand, 41BB ligand (4-1BBL), nerve growth factor and TNF-related apoptosis inducing ligand (TRAIL). In some embodiments of the present invention, the chimeric accessory molecule ligand genes of the present invention contain at least a portion of a murine accessory molecule ligand gene together with portions of accessory molecule ligand genes derived from either mouse, humans or other species. Some preferred embodiments of the present invention utilize murine CD40 ligand genes and chimeric CD40 ligand genes containing at least a segment of the murine CD40 ligand gene together with at least a segment of the human CD40 ligand gene. The present invention contemplates chimeric accessory molecule ligand genes wherein segments from the accessory molecule ligand gene of one species have been interchanged with segments from a second accessory molecule ligand gene which may optionally be from a different species. For example, in one preferred embodiment, the murine CD40 ligand gene

transmembrane and cytoplasmic domains have been attached to the extracellular domains of human CD40 ligand gene.

The present invention contemplates gene therapy vectors which are capable of directly infecting the human, mammal, insect, or other cell. The use of such gene therapy vectors greatly simplifies inserting an accessory molecule ligand gene into those cells. The contemplated gene therapy vectors may be used in vivo or in vitro to infect the desired cell and are particularly useful for infecting malignant cells to effect sustained high-level expression of a physiologic ligand.

The present invention also contemplates animal, mammal, and human cells containing a gene therapy vector which includes an accessory molecule ligand gene and sufficient genetic information to express that accessory molecule ligand within that cell. In preferred embodiments, the present invention also contemplates human neoplastic antigen presenting cells which contain the gene therapy vectors of the present invention or contain an accessory molecule ligand gene together with a promoter and 3′ end region.

The present invention also contemplates human cells and human neoplastic cells containing a gene therapy vector which includes a chimeric accessory molecule ligand gene. The present invention also contemplates bacterial cells or animal cells containing accessory molecule ligand genes, chimeric accessory molecule ligand genes, murine accessory molecule ligand genes, human accessory molecule ligand genes, the gene therapy vectors of the present invention, the vectors of the present invention, and a chimeric accessory molecule ligand gene together with a heterologous promoter, enhancer or polyadenylation sequence.

The present invention also contemplates methods of altering immune response within a human patient or the immunoreactivity of human cells in vivo by introducing a gene which encodes an accessory molecule ligand gene

into the human cells so that that accessory molecule ligand is expressed on the surface of those human cells. This method includes the introduction of the accessory molecule ligand gene as part of a gene therapy vector or in association with a heterologous or native promoter, enhancer or polyadenylation signal. Some preferred embodiments of the present invention utilize introduction of Fas ligand genes and chimeric Fas ligand genes, constructed as contemplated above for CD40, into human cells to alter their immunoreactivity. The present invention also includes methods in which such accessory molecule ligand genes are inserted into cells which have the accessory molecule to which the accessory molecule ligand binds on the surface of the cell into which the accessory molecule ligand gene.

The present methods of altering immunoreactivity are applicable to all types of human, animal, and murine cells including human neoplastic cells such as human lymphomas, leukemias and other malignancies. In preferred embodiments, this method is used to introduce the gene encoding the accessory molecule ligand into potential antigen presenting cells of a human patient or cell which can stimulate bystanding antigen presenting cells. Such antigen presenting cells include monocytes, macrophages, B cells, Langerhans cells, interdigitating cells, follicular dendritic cells, Kupffer cells, and the like. The various antigen presenting cells may be present as part of a known malignancy in a human patient such as leukemias, lymphomas, acute monocytic leukemia (AML), chronic lymphocytic leukemia (CLL), acute myelomonocytic leukemia (AMML), chronic myelogenous or chronic myelomonocytic leukemia (CMML) and thus would include all tumors of any cell capable of presenting antigen to the human or animal immune system or are capable of stimulating bystanding antigen presenting cells. The present invention also contemplates modulating the immune system by introducing genes

encoding an accessory molecule ligand gene of the present invention into any number of different cells found in a patient, including muscle cells, skin cells, stromal cells, connective tissue cells, fibroblasts and the like.

The present invention also contemplates methods of treating neoplasias in either a human patient or an animal patient. In one preferred embodiment, the method comprises isolating the neoplastic cells from the human or animal patient and inserting into those isolated cells the gene which encodes the chimeric accessory molecule ligand or the accessory molecule ligand so that that molecule is expressed on the cell surface of those neoplastic cells or other somatic cells. The neoplastic cells are then infused back into the human or animal patient and may then participate in an enhanced immune response.

The present invention also contemplates the co-infection or co-introduction of the accessory molecule ligand gene together with a gene which encodes a tumor or carcinoma specific antigen. This combination of molecules are then expressed on the surface of the neoplastic cells and when those cells are introduced into the patient lead to the rapid immune response resulting in the destruction of those cells.

The present methods also include directly introducing the gene therapy vector or other vector carrying the accessory molecule ligand gene directly into the tumor or tumor bed of a patient. Upon entering the tumor bed of the patient, the gene therapy vector or other vector enter the cells present in the tumor or tumor bed and then express the accessory molecule ligand gene on the surface of those cells. These cells then are able to participate fully in the human immune or animal immune response.

The present invention also contemplates methods of augmenting an immune response to a vaccine. The present

method of vaccinating an animal against a predetermined organism or antigen by administering to that animal a vaccine which has a genetic vector containing an accessory molecule ligand gene. Other embodiments of the present invention include vaccinating an animal by administering two separate genetic vectors, one containing the antigens from the organism to which immunity is desired by isolating the cells of the target animal and contacting with those cells a vector encoding at least one antigen from a predetermined organism so that the antigen is expressed by the cells and also contacting those cells with a different vector which expresses the accessory molecule ligand gene on the surface of the animal's antigen presenting cells. Together these two separate vectors produce a vaccination which is much stronger and of longer duration than is vaccination with antigen alone.

The present methods of vaccination are applicable to vaccinations designed to produce immunity against a virus, a cell, a bacteria, any protein or a fungus. The present methods are also applicable to immunization against various carcinomas and neoplasias. In these embodiments, the tumor antigen against which immunity is desired is introduced into the animal together with the genetic vector containing the accessory molecule ligand gene.

The present invention also contemplates methods of treating arthritis utilizing a gene therapy vector encoding an accessory molecule ligand. Of particular interest for use with arthritis is the Fas ligand molecule in which the expression of Fas ligand activity has been increased in the joint and/or the stability of the Fas ligand activity on cells within the joint enhanced. In other embodiments, the present invention contemplated methods of treating arthritis utilizing chimeric accessory molecule ligands and chimeric accessory molecule ligand genes. The present invention

also contemplates both ex vivo therapy and in vivo therapy of arthritis utilizing the expression vectors of the present invention together with the Fas ligand and modified versions of that molecule including chimeric molecules.

Brief Description of the Drawings

Figure 1. Figure 1 is a diagram showing a number of accessory molecule ligand genes and Domains I-IV of those genes as deduced from sequence data.

Figure 2. Figure 2 is a diagram showing example chimeric accessory molecule ligand genes. The domains derived from the murine accessory module are shown shaded.

Figure 3. Figure 3 shows the amount of either mouse or human CD40 ligand found on the surface of Hela or CLL cells infected with gene therapy vectors containing the genes encoding these molecules. Figure 3A shows uninfected Hela cells (shaded) and Hela cells infected with a gene therapy vector encoding murine CD40 ligand. Figure 3B shows uninfected Hela cells (shaded) and Hela cells infected with a gene therapy vector encoding human CD40 ligand. Figure 3C shows uninfected CLL cells (shaded) and CLL cells infected with a gene therapy vector encoding murine CD40 ligand. Figure 3D shows uninfected CLL cells (shaded) and CLL cells infected with a gene therapy vector encoding human CD40 ligand.

Figure 4. Figure 4 shows histograms of the increased expression of CD54 (Figure 4B) and CD80 (Figure 4D) on CLL cells into which a gene therapy vector containing the accessory molecule ligand gene (murine CD40 ligand gene) has been introduced. The shaded graph indicates control stain in FACS analysis and the open graph indicates staining with monoclonal antibodies immunospecific for either CD54 (Figures 4A and 4B) or CD80 (Figures 4C and 4D).

Figure 5. Figure 5 shows the cell proliferation as measured by ^{3}H-TdR incorporation of allogeneic T cells in response to various stimulation regimes. The CLL cells containing a gene therapy vector expressing an accessory molecule ligand gene (the murine CD40 ligand gene) were introduced, stimulating allogeneic T cells to proliferate.

Figure 6. Figure 6 shows the production of gamma interferon (IFNg) by allogeneic T cells stimulated with CLL cells containing an accessory molecule ligand gene.

Figure 7. Figure 7 shows the treatment of a neoplasia in an animal using a gene therapy vector containing an accessory molecule ligand gene of the present invention. The open squares show mice immunized with neoplastic cell not expressing an accessory molecule ligand of the present invention. Mice immunized with neoplastic cells expressing an accessory molecule ligand of the present invention are shown as the horizontal line at the top of the Figure and show no morbidity.

Figure 8. Figure 8 shows the production levels and stabilities of CD40 ligand and CD40 ligand transcript in CLL (upper graph) and normal blood mononuclear cells (lower graph).

Figure 9. Figure 9 shows the time course of transgene expression in CLL B cells infected with the accessory molecule ligand (CD40 ligand). The MFIR (mean fluorescence intensity ratio), comparing the fluorescence intensity of CD19^{+} CLL cells stained with PE-labeled CD40 ligand versus the same stained with a PE-labeled isotype control mAb at each time point, are represented by the closed circles connected by solid lines according to the scale provided on the left-hand ordinate.

Figure 10. Figure 10 shows changes in surface antigen phenotype of CLL B cells infected with a gene therapy vector containing an accessory molecule ligand,

CD40 ligand. Shaded histograms represent staining of uninfected CLL cells (thin lines) stained with nonspecific control antibody, open histograms drawn with thin lines represent uninfected CLL cells stained with FITC-conjugated specific mAb, and open histograms drawn with thick lines (labeled CD154-CLL) represent CLL cells infected with the accessory molecule ligand gene therapy vector and stained with FITC-conjugated specific mAb.

Figure 11. Figure 11 shows levels of CD27 produced in CLL cells infected with a gene therapy vector containing an accessory molecule ligand. Figure 11A shows that CD40L-infected CLL (CD154-CLL) cells express reduced levels of surface CD27. Open histograms represent staining of non-infected CLL cells (thin lines) or infected CLL (thick lines) with FITC-conjugated aCD27 mAb, respectively. Figure 11B shows production of soluble form of CD27 by CLL B cells.

Figure 12. Figure 12 shows allogeneic T cell responses induced by CLL cells infected with a gene therapy vector containing an accessory molecule ligand (CD40 ligand, also called CD154). Figure 12A indicates the concentration of IFNg in the supernatants after stimulation of allogeneic T cells with CLL cells containing the accessory molecule ligand. Figure 12B shows cell proliferation, as assessed by incorporation of ^{3}H-thymidine. Figures 12C and 12D show secondary allogeneic T cell responses induced by CLL containing the accessory molecule ligand.

Figure 13. Figure 13 depicts autologous T cell responses induced by CLL B cells containing the accessory molecule ligand, CD40 ligand or CD154, and controls. Figure 13A shows incorporation of ^{3}H-thymidine by autologous T cells co-cultured with the CLL cells. Figure 13B shows the levels of human IFNg produced by autologous T cells co-cultured with the CLL cells. In Figure 13C, the CTL activities of autologous T cells

induced by CLL B cells containing the accessory molecule ligand are graphed.

Figure 14. Figure 14 shows specificity of CTL for autologous CLL B cells. IFNg concentration was assessed in the supernatants after 48 h of culture (Figure 14A), and cytolytic activity was assessed at 3 h of culture (Figure 14B). In Figure 14C, mAb were added to the autologous leukemia target cells prior to the CTL assay.

Figure 15. Figure 15 shows that intercellular stimulation plays a role in production of the phenotypic changes observed in CLL cells expressing the accessory molecule ligand. In Figure 15A, the effect of culture density on the induced expression of CD54 and CD80 following infection with a gene therapy vector containing the accessory molecule ligand (CD40 ligand, CD154) is shown. Shaded histograms represent staining of leukemia B cells with a FITC-conjugated isotype control mAb. Open histograms represent CD154-CLL B cells, cultured at high or low density (indicated by arrows), and stained with a FITC-conjugated mAb specific for CD54 or CD80. Figure 15B shows inhibition of CD154-CLL cell activation by anti-CD154 mAb. Figures 15C and 15D depict expression of immune accessory molecules on bystander non-infected CLL B cells induced by CLL cells expressing the accessory molecule ligand. Shaded histograms represent staining with PE-conjugated isotype control mAb.

Figure 16. Figure 16 shows that the vector encoding an accessory molecule ligand enhances immunization against β-gal in mice. Figure 16A shows that mice that received intramuscular injections of the pCD40L vector produced significantly more antibodies to β-gal than did mice injected with either the non-modified pcDNA3 vector or pCD40L. Figure 16B, ELISA analyses of serial dilutions of sera collected at d28, shows that mice co-injected with placZ and pCD40L had an

eight-fold higher mean titer of anti-β-gal antibodies at d28 than mice treated with placZ + pcDNA3.

Figure 17. Figure 17 shows analysis of the IgG_1 and IgG_{2a} immune responses to intramuscular plasmid DNA immunizations with and without a vector, pCD40L, encoding an accessory molecule ligand. IgG_{2a} anti-β-gal antibodies predominated over IgG_1 subclass antibodies in the sera of mice injected with either placZ and pcDNA3 or placZ and pCD40L. In contrast, BALB/c mice injected with β-gal protein developed predominantly IgG_1 anti-β-gal antibodies, and no detectable IgG_{2a} anti-β-gal antibodies.

Figure 18. Figure 18 shows the comparison between injection of mice with a vector, pCD40L, encoding an accessory molecule ligand, at the same and different sites as placZ. Adjuvant effect of pCD40L requires co-injection with placZ at the same site.

Figure 19. Figure 19 shows that co-injection into dermis of a vector encoding an accessory molecule ligand, pCD40L, with placZ enhances the IgG anti-β-gal response in BALB/c mice.

Figure 20. Figure 20 shows that a vector encoding an accessory molecule ligand, pCD40L, enhances the ability of placZ to induce CTL specific for syngeneic b-gal-expressing target cells. Splenocyte effector cells, taken from mice which had received injections of placZ and pCD40L, specifically lysed significantly more cells than did splenocytes from mice that received control injections.

Figure 21. Figure 21 shows downmodulation of human CD40L, but not murine CD40L, in lung tumor cell lines that express CD40.

Figure 22. Figure 22A shows that CD40 binding induces enhanced expression of the tumor cell surface markers CD95 (Fas), CD54 (ICAM-1), and MHC-I, in lung tumor cell lines. Figure 22B shows downmodulation of human CD40L by CD40-positive tumor cells.

Figure 23. Figure 23 shows the inhibition of Fas ligand expression by lymphocytes in the presence of RA synovial fluid.

Figure 24. Figure 24 shows an outline for a clinical trial of an accessory molecule ligand (CD40L) gene therapy treatment for B cell CLL.

Figure 25. Figure 25 shows a sequence line-up of human Fas ligand with human Fas ligand in which Domain III is replaced by Domain III of murine Fas ligand. The top protein sequence is native human Fas ligand. Domain III is underlined with the dotted line. The double underline indicates a putative MMP cleavage site. The bottom protein sequence is that of chimeric human-mouse Fas ligand. Domain III of the mouse Fas ligand (underlined with dotted line) is substituted for Domain III of human Fas ligand. The numbers correspond to the amino acid sequence number using 1 for the start of the polypeptide sequence. The number of the first nucleotide base for the codon encoding the amino acid is 1+3x(n-1), where n is the amino acid sequence number.

Figure 26. Figure 26 shows a sequence line-up of human Fas ligand with human Fas ligand in which Domain III has been replaced with Domain III of human CD70. The top protein sequence is native human Fas ligand, and the bottom sequence is that of chimeric Fas ligand, in which Domain III of human CD70 has been substituted for Fas Domain III. Other markings are used similarly as in Figure 25.

Figure 27. Figure 27 shows a sequence line-up of human Fas ligand with human Fas ligand in which Domain I has been replaced with Domain III of human CD70. The top protein is native human Fas ligand, and the bottom protein sequence is that of chimeric Fas ligand, in which Domain III has been replaced with Domain I of human CD70. Other markings are used similarly as in Figure 25.

Figure 28. Figure 28 shows the amino acids around and at known matrix metalloproteinase (MMP) cleavage sites, as described in Smith, M.M. et al., Journal of Biol. Chem. 270:6440-6449 (95) and Nagase, H., and G.B. Fields, Biopolymers (Peptide Science) 40:399-416 (96). The cleavage site is indicated with an arrow.

Detailed Description of the Invention

All references cited herein are hereby incorporated in their entirety by reference.

I. Definitions

An "accessory molecule ligand gene" is a gene which encodes all or part of an accessory molecule ligand. The gene comprises at least the nucleotide sequence required to encode the functional portion of an accessory molecule ligand. The gene may optionally include such genetic elements as promoters, enhancers and 3' ends. The accessory molecule ligand gene is derived from a ligand which is a member of the tumor necrosis factor (TNF) family, including CD40 ligand, Fas ligand, CD70, TNF_{α}, TNF_{β}, CD30 ligand, 4-1BB ligand (4-1BBL), nerve growth factor and TNF-related apoptosis inducing ligand (TRAIL). As used herein, the term "accessory molecule ligand gene" includes chimeric accessory molecule ligand genes as defined below.

As used herein, the term "malignant cells or neoplastic cells," is defined to mean malignant or cancerous cells which are found in a human patient or an animal. Preferred types of malignant or neoplastic cells include any malignant antigen-presenting cell. In some preferred embodiments, these malignant antigen presenting cells have at least low levels of CD40 present on the cell surface.

As used herein, the term "neoplastic human cells" is defined to mean human cells which are neoplastic including but not limited to antigen presenting cells,

any neoplastic cell which may function as an antigen presenting cell or function to facilitate antigen presentation, neoplastic monocytes, neoplastic macrophages, neoplastic B cells, neoplastic dendritic cells, neoplastic Langerhans cells, neoplastic interdigitating cells, neoplastic follicular dendritic cells, or neoplastic Kupffer cells and the like. The definition of neoplastic human cells includes those cells which are associated with neoplastic cells in the tumor bed of human patients. Typically, the neoplastic human cells are either leukemias, lymphomas, AML, ALL, AMML, CML, CMML, CLL other tumors of antigen presenting cells or breast, ovarian or lung neoplastic cells. It is also contemplated that the accessory molecule ligand genes or chimeric accessory molecule ligand genes of the present invention may be inserted into somatic cells. These somatic cells can be created by a genetic engineering process which has introduced into those cells genes which encode molecules which render those cells capable of presenting antigen to the immune system.

As used herein, the term "chimeric gene" is defined to mean a gene in which part of the gene is derived from a second different gene and combined with the first gene so that at least a portion of each gene is present in the resulting chimeric gene. A gene may be chimeric if any portion of the sequence which encodes the resulting protein is derived from a second and different gene. Typical chimeric genes include genes in which specific functional domains from one gene have been transferred to a second gene and replace the analogous domains of that second gene. For example, the resulting chimeric gene may have one domain derived from a murine gene and several domains derived from a human gene. These domains may range in size from 5 amino acids to several hundred amino acids. Other examples of chimeric accessory molecule ligand genes include genes which

contain nucleotides encoding amino acids not found in any naturally occurring accessory molecule ligand gene. Examples of chimeric genes and potential various combinations of domains are numerous and one of skill in the art will understand that no limit is placed on the amount of one gene that must be present in a second gene to render it chimeric.

As used herein, the term "murine CD40 ligand gene" is defined to mean an accessory molecule ligand gene which is derived from a murine CD40 ligand gene. Examples of such murine CD40 ligand genes include the gene isolated by Armitage et al., Nature, 357:80-82 (1992) and other genes derived from murine origin which hybridize to the gene described by Armitage et al. under low stringency hybridization conditions.

As used herein, the term "vector or genetic vector" is defined to mean a nucleic acid which is capable of replicating itself within an organism such as a bacterium or animal cell. Typical genetic vectors include the plasmids commonly used in recombinant DNA technology and various viruses capable of replicating within bacterial or animal cells. Preferred types of genetic vectors includes plasmids, phages, viruses, retroviruses, and the like.

As used herein, the term "gene therapy vector" is defined to mean a genetic vector which is capable of directly infecting cells within an animal, such as a human patient. A number of gene therapy vectors have been described in the literature, and include, the gene therapy vector described in Cantwell et al., Blood, In Press (1996) entitled "Adenovirus Vector Infection of Chronic Lymphocytic Leukemia B Cells." Such vectors have been described for example by Woll, P. J. and I. R. Hart, Ann. Oncol., 6 Suppl 1:73 (1995); Smith, K. T., A. J. Shepherd, J. E. Boyd, and G. M. Lees, Gene Ther., 3:190 (1996); Cooper, M. J., Semin. Oncol., 23:172 (1996); Shaughnessy, E., D. Lu, S. Chatterjee, and K. K.

Wong, Semin. Oncol., 23:159 (1996); Glorioso, J. C., N. A. DeLuca, and D. J. Fink, Annu. Rev. Microbiol., 49:675 (1995); Flotte, T. R. and B. J. Carter, Gene Ther., 2:357 (1995); Randrianarison-Jewtoukoff, V. and M. Perricaudet, Biologicals., 23:145 (1995); Kohn, D. B., Curr. Opin. Pediatr., 7:56 (1995); Vile, R. G. and S. J. Russell, Br. Med. Bull., 51:12 (1995); Russell, S. J., Semin. Cancer Biol., 5:437 (1994); and Ali, M., N. R. Lemoine, and C. J. Ring, Gene Ther., 1:367 (1994). All references cited herein are hereby incorporated by reference.

II. Genetic Vectors and Constructs Containing an Accessory Molecule Ligand Gene

A. Accessory Molecule Ligand Genes

In one embodiment of the present invention, preferred gene therapy vectors contain an accessory molecule ligand gene. This accessory molecule ligand gene may be derived from any source and may include molecules which are man-made and do not appear in nature. The present invention contemplates accessory molecule ligand genes which are derived from the genes encoding molecules within the tumor necrosis family (TNF) which includes the genes encoding: murine CD40 ligand, human CD40 ligand, Fas ligand, TNF_α, TNF_β, CD30 ligand, 4-1BB ligand, nerve growth factor, CD70, TNF-related apoptosis inducing ligand (TRAIL) and chimeric accessory molecule ligands. The nucleotide sequence of one accessory molecule ligand, the sequence of at least one form of the murine CD40 ligand gene, has been determined and is listed as SEQ ID NO: 1. The present invention contemplates the use of any accessory molecule ligand gene which is homologous to the sequence present in SEQ ID NO: 1, and thus hybridizes to this sequence at low stringency hybridization conditions. One of skill in the art will understand that accessory molecule ligand genes, including murine CD40 ligand gene, useful

in the present invention may be isolated from various different murine strains.

The nucleotide sequence of a human CD40 ligand gene has been determined and is shown as SEQ ID NO: 2. The present invention contemplates the use of any accessory molecule ligand gene which is homologous to SEQ ID NO: 2, and thus hybridizes to this sequence at low stringency conditions. One of ordinary skill in the art will understand that the accessory molecule ligand genes, including the human CD40 ligand genes, useful in the present invention, may vary depending on the individual from which the gene is isolated and such variations may prove useful in producing unique accessory molecule ligand genes. The present invention contemplates the use of the domains, sub-domains, amino acid or nucleotide sequence of the human CD40 ligand and/or human CD40 ligand gene as part of a chimeric accessory molecule ligand or chimeric accessory molecule ligand gene.

The nucleotide sequence of a bovine CD40 ligand gene has been determined and is shown as SEQ ID NO: 8. The present invention contemplates the use of any accessory molecule ligand gene which is homologous to SEQ ID NO: 8, and thus hybridizes to the sequence at low stringency conditions. One of ordinary skill in the art will understand that the accessory molecule ligand genes, including the bovine CD40 ligand genes, may vary depending on the individual animal from which the gene is isolated and that such variations may prove useful in producing unique accessory molecule ligand genes.

The nucleotide sequence of human TNF_α and human TNF_β have been determined and are shown as SEQ ID NOS: 9 and 10, respectively. The present invention contemplates the use of any accessory molecule ligand gene which is homologous to either human TNF_α or human TNF_β (SEQ ID NOS: 9 and 10, respectively), and thus hybridizes to these sequences at low stringency conditions. The

accessory molecule ligand genes useful in the present invention, including the human TNF_{α} and TNF_{β} genes, may vary depending on the particular individual from which the gene has been isolated and these variations may prove useful in producing unique accessory molecule genes.

The nucleotide sequence of porcine TNF_{α} and TNF_{β} have been determined and are shown as SEQ ID NO: 11. The present invention contemplates the use of any accessory molecule ligand gene which is homologous to either SEQ ID NO: 11, and thus would hybridize to these sequences at low stringency conditions. One of ordinary skill in the art will understand that the accessory molecule ligand genes, including the porcine TNF_{α} and TNF_{β} genes, may vary depending on the particular animal from which the gene is isolated and that such variation may prove useful in producing unique accessory molecule genes.

The nucleotide sequence of a murine TNF_{α} gene has been determined and is shown as SEQ ID NO: 12. The present invention contemplates the use of any accessory molecule ligand gene which is homologous to SEQ ID NO: 12, and thus hybridizes to the sequence at low stringency conditions. One of ordinary skill in the art will understand that the accessory molecule ligand genes, including the murine TNF_{α} gene may vary depending on the individual from which the gene is isolated and that these variations may prove useful in producing unique accessory molecule genes.

The nucleotide sequence of human Fas ligand and murine (C57BL/6) Fas ligand have been determined and are shown as SEQ ID NOS: 13 and 14, respectively. The nucleotide sequence of murine Balb/c Fas ligand is shown as SEQ ID NO: 31. The present invention contemplates the use of any accessory molecule ligand gene which is homologous to any of SEQ ID NOS: 13, 14, and 31, and thus hybridizes to the sequences at low stringency

conditions. One of ordinary skill in the art will understand that the accessory molecule ligand genes, including the human Fas ligand or murine Fas ligand genes may vary depending on the particular individual or animal from which the gene is isolated and that such variations may prove useful in producing any accessory molecule genes.

The nucleotide sequence of a human CD70 gene has been determined and is shown as SEQ ID NO: 15. The murine CD70 gene sequence has also been determined, and is shown as SEQ ID NO: 36 and was described by Tesselaar et. al, J. Immunol. 159:4959-65(1997). The present invention contemplates the use of any accessory molecule ligand gene which is homologous to SEQ ID NO: 15 or 36, and thus hybridizes to this sequence at low stringency conditions. One of ordinary skill in the art will understand that the accessory molecule ligand genes, including the human CD70 gene may vary depending on the individual from which the gene is isolated and that these variations may prove useful in producing unique accessory molecule ligand genes.

The nucleotide sequence of human CD30 ligand gene has been determined and is shown as SEQ ID NO: 16. The present invention contemplates the use of any accessory molecule ligand gene which is homologous to SEQ ID NO: 16, and thus hybridizes to this sequence at low stringency conditions. One of ordinary skill in the art will understand that the accessory molecule ligand genes, including the human CD30 ligand gene, may vary depending on the individual from which the gene is isolated and that such variations may prove useful in producing unique accessory molecule ligand genes.

The present invention also contemplates variations and variants of the nucleotide sequences of the accessory molecule ligand genes provided herein which are caused by alternative splicing of the messenger RNA. This alternative splicing of the messenger RNA inserts

additional nucleotide sequences which may encode one or more optional amino acid segments which in turn allows the accessory molecule ligand encoded to have additional properties or functions.

The nucleotide sequence of a human and mouse 4-1BBL have been determined and are shown as SEQ ID NOS: 17 and 18, respectively. The present invention contemplates the use of any accessory molecule ligand gene which is homologous to either SEQ ID NOS: 17 or 18, and thus hybridizes to these sequences at low stringency conditions. One of ordinary skill in the art will understand that accessory molecule ligand genes, including the human 4-1BBL gene may vary depending on the individual from which it is isolated and that such variations may prove useful in producing unique accessory molecule ligand genes.

The present invention also contemplates chimeric accessory molecules containing any domain, sub-domain portion, or amino acid sequence encoded by the following genes: bovine TNF-α (SEQ ID NO: 21), murine CD40 ligand (SEQ ID NO: 22), human nerve growth factor-β (SEQ ID NO: 23), murine nerve growth factor (SEQ ID NO: 24), rat Fas ligand (SEQ ID NO: 25), human TNF-related apoptosis inducing ligand (TRAIL) (SEQ ID NO: 41, Genbank accession number U37518), murine TNF-related apoptosis inducing ligand (TRAIL) (SEQ ID NO: 42, Genbank accession number U37522), murine CD30-Ligand (SEQ ID NO: 43), human 4-1BBL (SEQ ID NO: 17), and murine 4-1BBL (SEQ ID NOS: 44 and 18). The present invention also contemplates chimeric accessory molecules which utilize genes encoding amino acid sequences homologous to these sequences.

The present invention contemplates chimeric accessory molecule ligand genes which are comprised of a nucleotide segment derived from one accessory molecule ligand gene operatively linked to a nucleotide sequence

derived from a different accessory molecule ligand gene or other gene.

For example, chimeric accessory molecule ligand genes are contemplated which are comprised of a segment of the murine CD40 ligand gene which has been operatively linked to at least one other additional gene segment derived from a different accessory molecule ligand gene. The size of the particular segment derived from the different accessory molecule ligand gene may vary from a nucleotide sequence encoding a few amino acids, a sub-domain of the accessory molecule ligand, a domain of the accessory molecule ligand or more than a domain of an accessory molecule ligand. Other chimeric accessory molecules of the present invention are comprised of an accessory molecule ligand gene into which nucleotides encoding an amino acid segment which is not found as part of a naturally occurring accessory molecule ligand have been inserted. This amino acid segment may be artificially created or derived from a protein found in nature. The chimeric accessory molecule ligand gene encodes a chimeric amino acid sequence and thus a chimeric accessory molecule ligand encoded may possess unique properties in addition to the properties found on the individual segments derived from the different accessory molecule ligand genes. The chimeric accessory molecule ligand gene may encode an accessory molecule ligand which has properties derived from the accessory molecule ligand used to construct the chimeric gene.

Each of the accessory molecule ligand genes which are a member of the tumor necrosis factor family have a similar secondary structure consisting of a number of domains. This domain structure includes a first domain which is encoded by the 5′ region of the accessory molecule ligand gene. The second domain (Domain II) is the domain which contains the amino acids which span the cell membrane and is thus called the transmembrane

domain. The third domain (Domain III) is the proximal extracellular domain and these amino acids are the amino acids which are found proximal to the cellular membrane. The fourth domain (Domain IV), is encoded by the 3′ end of the accessory molecule ligand gene and has been called the distal extracellular domain. The distal extracellular domain (Domain IV) generally makes up the soluble form of the tumor necrosis factor family molecule. Based on the x-ray crystal structure of human TNF, the predicted secondary structure of the accessory molecule, CD40 ligand has been deduced together with the domain structure of these molecules by M. Peitsch and C. Jongeneel, International Immunology, 5:233-238 (1993). The secondary structures of the other members of the tumor necrosis factor family were deduced using computer analysis together with comparison to the human TNF and CD40 ligand domain structure. In Table I, the domain boundaries of a number of accessory molecule ligand genes is shown. A diagram of these domains for a number of these accessory cell molecule ligands is shown in Figure 1. The assignments of the domain boundaries are approximate and one of ordinary skill in the art will understand that these boundaries may vary and yet still provide useful identification of domains.

TABLE I

DOMAIN STRUCTURE OF TUMOR NECROSIS FACTOR FAMILY MOLECULES*

	Domain I (Cytoplasmic)	Domain II (Transmembrane)	Domain III (Proximal Extracellular)	Domain IV (Distal Extracellular)
Human CD40 Ligand	1-42	43-135	136-330	331-786
Murine CD40 Ligand	1-42	43-135	136-327	328-783
Bovine CD40 Ligand	1-42	43-135	136-330	331-786
Human TNF-α	1-87	88-168	169-228	229-699
Murine TNF-α	1-87	88-168	169-237	238-705
Porcine TNF-α	1-87	88-168	169-228	229-696
Human TNF-β	1-39	40-129	130-153	154-615
Porcine TNF-β	1-39	40-126	127-150	151-612
Human Fas Ligand	1-237	238-315	316-390	391-843
Murine Fas Ligand	1-237	238-309	310-384	385-837
Human CD70	1-61	62-117	118-132	133-579
Murine CD70	1-73	74-123	124-138	139-585
Human CD30 Ligand	1-117	118-186	187-240	241-702
Murine CD30 Ligand	1-135	136-201	202-255	256-717
Human 4-1BBL	1-69	70-174	175-210	211-762
Murine 4-1BBL	1-237	238-333	334-369	370-927
Human TRAIL	1-39	40-117	118-375	376-843
Murine TRAIL	1-51	52-111	112-387	388-873

* The Domains above are identified by the nucleotide boundaries of each domain using the first nucleotide of the initial methionine of the cDNA as nucleotide number 1.

One of ordinary skill in the art will understand that typical chimeric accessory molecule genes would include genes produced by exchanging domains or sub-domain segments between, for example, a mouse CD40 ligand gene and a human CD40 ligand gene. For example, chimeric accessory molecule gene may be constructed by operatively linking Domain I of the human CD40 ligand gene to Domains II-IV of the murine CD40 ligand gene. One of ordinary skill in the art will understand the variety of chimeric accessory molecule ligand genes which may be produced using the accessory molecules identified in Table I. The present invention also contemplates chimeric accessory molecules which are not shown in Table I but which are shown to have a similar domain structure. Other chimeric genes are also contemplated in which smaller segments (sub-domain segments) are exchanged between, for example, a murine CD40 ligand gene and a human CD40 ligand gene or a second murine CD40 ligand gene. One of skill in the art will understand that genes encoding accessory molecules will have at least gene segments which correspond to various functional segments of an accessory molecule ligand such as the murine CD40 ligand encoded by the murine CD40 ligand gene (SEQ ID NO: 1). It will also be apparent to one of skill in the art that the nucleotide boundaries identified in Table I may vary considerably from those identified for the murine CD40 ligand gene (SEQ ID NO: 1) and still define domains which are useful in the present invention.

In one preferred embodiment, the chimeric accessory molecule ligand gene is comprised of the nucleotides encoding extracellular domains (Domains III and IV) of human CD40 ligand operatively linked to the nucleotides encoding transmembrane (Domain II) and the nucleotides encoding cytoplasmic domain (Domain I) of the murine CD40 ligand gene. Examples of such preferred chimeric accessory molecules are shown in Figure 2. An exemplary

nucleotide sequence for such a gene is SEQ ID NO: 7. In other chimeric accessory molecule ligand genes of the present invention, the nucleotides encoding the extra-cellular domains (Domains III and IV) of the murine CD40 ligand gene may be operatively linked to nucleotides encoding the transmembrane (Domain II) and cytoplasmic domain (Domain I) of the human CD40 ligand gene. An exemplary nucleotide sequence for such a gene is SEQ ID NO: 3. In other preferred chimeric accessory molecule ligand genes of the present invention, the nucleotides encoding the extracellular domains (Domains III and IV) and transmembrane domain (Domain II) of human CD40 ligand are coupled to the nucleotides encoding cyto-plasmic domain (Domain I) of murine CD40 ligand gene. An exemplary nucleotide sequence for such a gene is SEQ ID NO: 6. Other chimeric accessory molecule genes contemplated by the present invention comprise the nucleotides encoding the extracellular domains (Domains III and IV) and transmembrane domain (Domain I) of the murine CD40 ligand gene operatively linked to the nucleotides encoding cytoplasmic domain of the human CD40 ligand gene. An exemplary nucleotide sequence for such a gene is SEQ ID NO: 5. Other chimeric accessory molecule ligand genes are contemplated by the present invention in which the human CD40 ligand gene extracellular domains (Domain III and IV) is operatively linked to the murine CD40 ligand gene transmembrane domain (Domain I) which is operatively linked to the human CD40 ligand gene cytoplasmic domain (Domain I). An exemplary nucleotide sequence for such a gene is SEQ ID NO: 4.

One of ordinary skill in the art will understand that many more combinations which utilize domains or other selected segments of any of the accessory molecule ligand genes including the human CD40 ligand genes and the mouse CD40 ligand genes are possible. Such additional chimeric accessory molecule genes would

include the following genes: chimeric accessory molecule genes in which the nucleotides encoding Domain I are selected from a particular accessory molecule ligand gene and operatively linked, either directly or by an additional nucleotide sequence to the nucleotides encoding Domain II from a particular accessory molecule ligand gene. These domains then would be operatively linked either directly or by an additional nucleotide sequence to the nucleotides encoding Domain III from a particular accessory molecule ligand gene. This molecule would then be operatively linked either directly or by an additional nucleotide sequence to the nucleotides encoding Domain IV of a particular accessory molecule ligand gene. The chimeric accessory molecule ligand gene constructed in this manner may have additional nucleotides on either end or between domains which are useful to provide different amino acids in these positions. One of ordinary skill in the art will understand that these particular combinations are merely illustrations and that numerous other combinations could be contemplated in which gene segments comprising nucleotides encoding less than the entire domain of an accessory molecule are exchanged between different accessory molecules.

The present invention also contemplates chimeric accessory molecule ligand genes which are comprised of gene segments of mouse or human CD40 ligand in combination with gene segments derived from Fas ligand, TNF_{α}, TNF_{β}, CD70, CD30L, 4-1BBL, nerve growth factor or TNF-related apoptosis inducing ligand (TRAIL). Particularly useful chimeric accessory molecule ligand genes comprise at least one gene segment which is derived from a murine CD40 ligand gene together with gene segments or a gene segments derived from a different accessory molecule ligand gene.

The present invention also contemplates chimeric accessory molecule ligand genes in which the accessory

molecules produced have been modified to remove amino acids within the chimeric accessory molecule that are used by post-translational mechanisms to regulate the level of expression of the accessory molecule or accessory molecule protein on a particular cell. The sites removed from the chimeric accessory molecules or chimeric molecule may include amino acids or sites which make up protease cleavage sites including metallothionine proteases, serine proteases and other proteases that recognize an amino acid sequence either specifically or nonspecifically. In particular preferred embodiments, amino acids in Domain III which make up potential or actual recognition site(s) used by post-translational regulatory mechanisms have been modified or removed.

The present invention also contemplates chimeric accessory molecule ligand genes in which the domains, subdomain fragments or other amino acid residues have been taken from one accessory molecule ligand gene and moved into a second accessory molecule ligand gene from the same species. For example, in this particular embodiment, the human Domain I, and the human Domain II from the CD40 ligand molecule may be operatively linked to the nucleotides encoding the human Domain III from, for example, the CD70 molecule which is in turn operatively linked to human Domain IV for the CD40 ligand molecule. This chimeric accessory molecule therefore contains human CD40L Domains I, II and IV and human CD70 Domain III. An exemplary nucleotide sequence for such a gene is SEQ ID NO: 19. One of ordinary skill in the art will understand that a number of such combinations using domains from the same species from different accessory molecule ligand genes may create a number of chimeric accessory molecule genes which may all have specific activities and properties.

The present invention contemplates chimeric accessory molecule ligand genes in which the Domain III

of a particular accessory molecule ligand gene has been replaced with a Domain III from a different accessory molecule ligand gene. In one particularly preferred embodiment, the mouse Domain III has been used to replace the human Domain III in the CD40 ligand molecule. This chimeric accessory molecule therefore contains the human CD40L Domain I, the human CD40L Domain II, mouse CD40L Domain III, and human CD40L Domain IV. An exemplary nucleotide sequence for such a gene is SEQ ID NO: 20.

The present invention also contemplates the use of chimeric accessory molecules that contain man-made amino acid sequences inserted into or in place of a portion of a domain or other amino acid sequence of an accessory molecule gene. These man-made amino acid segments may be created by selecting any amino acid sequence that may be used to give the accessory molecule a particular function or to remove another undesired function. These man-made amino acid segments are produced by inserting into the accessory molecule ligand gene or chimeric accessory molecule ligand gene the nucleotide sequences required to encode those particular man-made amino acid segments in the desired positions. Further, the chimeric accessory molecule ligand genes may contain nucleotide segments which comprise sub-domain segments of other molecules or small segments in which amino acids have been changed for a desired purpose. The use of sub-domain nucleotide segments allows the introduction of short amino acid sequences derived from other molecules into chimeric accessory molecules of the present invention. The incorporation of such short sub-domain segments or amino acid changes into the accessory molecule ligand allows the introduction of desired or the removal of undesired features of that molecule.

The identification of domain structures within accessory cell molecules is well known in the art and generally requires the identification of cysteine

residues within the accessory molecules and the subsequent mapping of disulfide bonds between various cysteine residues. The mapping of various sub-domain segments of an accessory molecule is well known in the art and involves analysis of the amino acid sequence of the accessory molecules and generally involves a comparison of the crystal structure of tissue necrosis factor with the use of predictive algorithms thereby producing a predicted structure of a chimeric accessory molecule or an accessory molecule. This predicted structure of these molecules can then be used to select various sub-domain portions of the molecule to be used to construct further chimeric accessory molecules. Examples of such mapping studies include the studies by M. Pitsch and C. V. Jongeneel, International Immunology, 5:233-238 (1993) and the analysis shown in Figure 1.

The present invention also contemplates accessory molecule ligand genes and chimeric accessory molecule ligand genes which are truncated and encode less than the full length of the amino acid sequence found in the native accessory molecule ligand. These truncations may alter the properties of the accessory molecule ligand gene but some identified activity is maintained. Such truncations may be made by removing a gene segment or gene segments from the accessory molecule gene and typically would be performed by removing nucleotides encoding domains which are not directly involved in the binding of the accessory molecule ligand with its accessory molecule. These truncated accessory molecule ligand genes or chimeric truncated accessory molecule ligand genes may contain further gene segments which encode amino acid segments or domains which replace the domains removed from that truncated accessory molecule gene. However, such replacement of the portions of the accessory molecule removed by truncation is not necessary.

The chimeric accessory molecule genes of the present invention may be constructed using standard genetic engineering methods to operatively link a particular nucleotide sequence from one accessory molecule ligand gene to a different nucleotide sequence derived from the same or different accessory molecule ligand gene. In addition, standard genetic engineering methods may be used to insert man-made nucleotide sequences or sub-domain nucleotide sequences into the chimeric accessory molecule ligand gene. One of ordinary skill in the art will understand that various methods may be utilized to produce such chimeric accessory molecule genes. For example, a gene conversion method known as "SOEN" may be used to produce a chimeric accessory molecule gene which contains nucleotide segments derived from different chimeric accessory molecules. The methods for using this gene conversion method are well known in the art and have been described for example in Horton, R. M., Mol. Biotechnol., 3:93 (1995); Ali, S. A. and A. Steinkasserer, Biotechniques, 18:746 (1995); Vilardaga, J. P., E. Di Paolo, and A. Bollen, Biotechniques, 18:604 (1995); Majumder, K., F. A. Fattah, A. Selvapandiyan, and R. K. Bhatnagar, PCR. Methods Appl., 4:212 (1995); Boles, E. and T. Miosga, Curr. Genet. 28:197 (1995); Vallejo, A. N., R. J. Pogulis, and L. R. Pease, PCR. Methods Appl., 4:S123 (1994); Henkel, T. and P. A. Baeuerle, Anal. Biochem., 214:351 (1993); Tessier, D. C. and D. Y. Thomas, Biotechniques, 15:498 (1993); Morrison, H. G. and R. C. Desrosiers, Biotechniques, 14:454 (1993); Cadwell, R. C. and G. F. Joyce, PCR. Methods Appl., 2:28 (1992); and, Stappert, J., J. Wirsching, and R. Kemler, Nucleic Acids Res., 20:624 (1992). Alternatively, one of ordinary skill in the art will understand that site-directed mutagenesis may be used to introduce changes into a particular nucleotide sequence to directly produce or indirectly be used to

produce a chimeric accessory molecule gene of the present invention. For example, the mutagen kit provided by BioRad Laboratories may be used together with the methods and protocols described within that kit to produce the desired changes in the nucleotide sequence. These methods were originally described by Kunkel, Proc. Natl. Acad. Sci. USA, 82:488-492 (1985) and Kunkel et al., Meth. Enzol. Mol., 154:367-382 (1987). By using the site directed mutagenesis protocols described herein and known within the art, a skilled investigator may induce individual nucleotide changes which result in an altered amino acid sequence or which preserve an amino acid sequence but introduce a desired restriction enzyme recognition sequence into the gene. This new restriction endonuclease recognition site may then be used to cut the gene at that particular point and use it to a gene or segment of another accessory molecule ligand gene. In addition to these methods, one of ordinary skill in the art will understand that an entire chimeric accessory molecule ligand gene may be synthesized using synthetic methods known in the art. This methodology only requires that the skilled artesian generating nucleotide sequence of a chimeric accessory molecule ligand gene and provide that sequence to a company which is capable of synthesizing such a gene.

B. Genetic Constructs

The present invention contemplates the use of accessory molecule ligand genes or chimeric accessory molecule ligand genes which are present in various types of genetic vectors. A genetic vector refers to a DNA molecule capable of autonomous replication in a cell into which another DNA segment can be inserted to cause the additional DNA segments to replicate. Vectors capable of expressing genes contained in that vector are referred to as "expression vectors." Thus, the genetic vectors and expression vectors of the present invention

are recombinant DNA molecules which comprise at least two nucleotide sequences not normally found together in nature.

The genetic vectors useful in the present invention contain an accessory molecule ligand gene which encodes an accessory molecule ligand which is optionally operatively linked to a suitable transcriptional or translational regulatory nucleotide sequence, such as one derived from a mammalian, microbial, viral, or insect gene. Such regulatory sequences include sequences having a regulatory role in gene expression, such as a transcriptional promoter or enhancer, an operator sequence to control transcription, a sequence encoding a ribosomal binding site within the messenger RNA and appropriate sequences which control transcription, translation initiation or transcription termination.

Particularly useful regulatory sequences include the promoter regions from various mammalian, viral, microbial, and insect genes. The promoter region directs an initiation of transcription of the gene and causes transcription of DNA through and including the accessory molecule ligand gene. Useful promoter regions include the promoter found in the Rous Sarcoma Virus (RSV) - long terminal repeat (LTR), human cytomegalovirus (HCMV) enhancer/promoter region lac promoters, and promoters isolated from adenovirus, and any other promoter known by one of ordinary skill in the art would understand to be useful for gene expression in eukaryotes, prokaryotes, viruses, or microbial cells. Other promoters that are particularly useful for expressing genes and proteins within eukaryotic cells include mammalian cell promoter sequences and enhancer sequences such as those derived from polyoma virus, adenovirus, simian virus 40 (SV40), and the human cytomegalovirus. Particularly useful are the viral early and late promoters which are typically found

adjacent to the viral origin of replication in viruses such as the SV40. Examples of various promoters which have been used in expression vectors have been described by Okiama and Berg (Mol. Cell. Biol. 3:280, 1983), the pMLSVN SV40 described by Kossman et al., Nature 312:768 (1984). One of ordinary skill in the art will understand that the selection of a particular useful promoter depends on the exact cell lines and the other various parameters of the genetic construct to be used to express the accessory molecule ligand gene or the chimeric accessory molecule ligand gene within a particular cell line. In addition, one of ordinary skill in the art will select a promoter which is known to express genes in the target cell at a sufficiently high level to be useful in the present invention.

The genetic vectors and expression vectors of the present invention optionally contain various additional regulatory sequences including ribosome binding sites which allow the efficient translation of the messenger RNA produced from an expression vector into proteins, the DNA sequence encoding various signals peptides which may be operatively linked to the accessory molecule ligand gene or the chimeric accessory molecule ligand gene. The signal peptide, if present, is expressed as a precursor amino acid which enables improved extra-cellular secretion of translation fusion polypeptide.

The genetic constructs contemplated by the present invention therefore include various forms of accessory molecule ligand genes described above which are operatively linked to either a promoter sequence or a promoter and enhancer sequence and also operatively linked to a polyadenylation sequence which directs the termination and polyadenylation of messenger RNA. It is also contemplated that the genetic constructs of the present invention will contain other genetic sequences which allow for the efficient replication and expression of that construct within the desired cells. Such

sequence may include introns which are derived from native accessory molecule ligand genes or, for example, from a virus gene.

The present invention also contemplates gene therapy vectors which are able to directly infect mammalian cells so as to introduce the desired accessory molecule ligand gene or chimeric accessory molecule ligand gene into that cell. These gene therapy vectors are useful for directly infecting cells which have been isolated from an animal or patient, or can be directly introduced into an animal or patient and thereby directly infect the desired cell within that animal or patient.

Many types of gene therapy vectors which are able to successfully transfer genes and cause the expression of desired foreign DNA sequences have been developed and described in the literature. For example, the article entitled "Gene Transfer Vectors for Mammalian Cells" in Current Comm. Mol. Biol., Cold Springs Harbor Laboratory, New York (1987). Further, naked DNA can be physically introduced into eukaryotic cells including human cells by transvection using any number of techniques including calcium phosphase transfection (Berman et al., Proc. Natl. Acad. Sci. USA, 81:7176 (1984)), DEAE-Dextran Transfection, protoplast fusion (Deans et al., Proc. Natl. Acad. Sci. USA, 81:1292 (1984)), electroporation, liposome fusion, polybrene transfection and direct gene transfer by laser micropuncture of the cell membrane. In addition, one of ordinary skill in the art will understand that any technique which is able to successfully introduce the DNA into a cell in such a manner as to allow it to integrate into the genome of a cell and allow the expression of the desired gene would be useful in the present invention.

Specifically, gene therapy vectors which utilize recombinant infectious virus particles for gene delivery

have been widely described. See, for example, Brody, S. L. and R. G. Crystal, Ann. N. Y. Acad. Sci., 716:90 (1994); Srivastava, A., Blood. Cells, 20:531 (1994); Jolly, D., Cancer Gene Ther., 1:51 (1994); Russell, S. J., Eur. J. Cancer, 30A:1165 (1994); Yee, J. K., T. Friedmann, and J. C. Burns, Methods Cell Biol., 43 Pt A:99 (1994); Boris-Lawrie, K. A. and H. M. Temin, Curr. Opin. Genet. Dev., 3:102 (1993); Tolstoshev, P., Annu. Rev. Pharmacol. Toxicol., 33:573 (1993); and, Carter, B. J., Curr. Opin. Biotechnol., 3:533 (1992). The present invention contemplates the use of gene therapy vectors to carry out the desired methodology of the present invention by introducing a gene encoding an accessory molecule ligand gene or a chimeric accessory molecule ligand gene into the cell. Many viral vectors have been defined and used as gene therapy vectors and include virus vectors derived from simian virus 40 (SV40), adenoviruses, adeno-associated viruses, and retroviruses. One of ordinary skill in the art will understand that useful gene therapy vectors are vectors which are able to directly introduce into the target cells the DNA which encodes the accessory molecule ligand and allow that DNA to persist in the cell so as to express the accessory molecule ligand in the desired manner within the cell.

The gene therapy vectors of the present invention are useful for introducing accessory molecule ligand genes into a variety of mammalian cells including human cells. The particular cells infected by the gene therapy vector will depend on the various specifics of the vector and such vectors can be used to introduce the accessory molecule ligand genes of the present invention into hematopoietic or lymphoid stem cells, antigen presenting cells, embryonic stem cells, and other cells which are capable of presenting antigen within the immune system including cells which have CD40 on their surface. Further, such gene therapy vectors are able to

introduce a gene encoding an accessory molecule ligand gene into a human neoplastic cell such as a lymphoma, leukemia, AML, CLL, CML, AMML, CMML, breast cancer, lung cancer, ovarian cancer or any tumor capable of acting as antigen presenting cells or cells which can stimulate bystander antigen presenting cells. Further, the contemplated gene therapy vectors may be used to introduce the accessory molecule ligand genes of the present invention into cells which have been engineered to make those cells capable of presenting antigen to the immune system.

III. Cells Containing Genetic Constructs Encoding an Accessory Molecule Ligand or Chimeric Accessory Molecule Ligand

The present invention also contemplates various cells which contain the genetic constructs of the present invention. These cells contain the constructs which encode the accessory molecule ligand gene and thus contain the various genetic elements described in Section II.B. above. These cells may be microbial cells, eukaryotic cells, insect cells, and various mammalian cells including human cells. In preferred embodiments of the present invention, these cells include various neoplastic cells including human neoplastic cells. These neoplastic cells may be of any cell type and include cells of the immune system, and other blood cells. Particularly preferred are any neoplastic cells which may function as an antigen presenting cells within the immune system or which may stimulate bystander antigen presenting cells by expression of a transgenic accessory cell molecule of the present invention. Typically these neoplastic which are able to function to present antigen to the immune system have or have had an accessory molecule, such as the CD40 molecule, on the cell surface. Generally, these cells are naturally capable of presenting antigen to the immune system, but the present invention also

contemplates the introduction of accessory molecule ligand genes into a cell which is not naturally able to present antigen to the immune system but which has been genetically engineered to make that cell capable of presenting antigen to the immune system. Typically, these cells include various known cell types such as monocytes, macrophages, B cells, Langerhans cells, interdigitating cells, follicular dendritic cells or Kupffer cells and the like which have become neoplastic. In addition, the present invention also contemplates cells from various carcinomas, breast, ovarian and lung cancers which contain the genetic constructs described herein. In other preferred embodiments, an accessory molecule ligand gene of the present invention is placed into cells which may be injected into a treatment site such as a tumor bed or joint. For example, the accessory molecule ligand gene of the present invention may be inserted into a fibroblast cell and the accessory molecule ligand expressed on the surface of that cell. The fibroblasts are then injected into the treatment site and cause the desired immuno effect due to the presence of the accessory molecule ligand on the surface of those cells. These cells stimulate other immune cells present in that treatment site (bystander cells). This process then results in the desired effect on the immune system.

IV. Methods Utilizing Genetic Vectors and Constructs Containing an Accessory Molecule Ligand Gene

The present invention contemplates methods of altering the immunoreactivity of human cells using a method which includes introducing a gene encoding an accessory molecule ligand gene into the human cells so that the accessory molecule ligand encoded by that gene is expressed on the surface of those cells. The present invention is useful for any human cells which participate in an immune reaction either as a target for

the immune system or as part of the immune system which responds to the foreign target. A large variety of methods are contemplated in which the final result is that the accessory molecule ligand gene is introduced into the desired cells. These methods include _ex vivo_ methods, _in vivo_ methods and various other methods which involve injection of DNA, genetic vectors or gene therapy vectors into the animal or human, including injection directly into the tumor bed present in any animal or human.

Ex vivo methods are contemplated wherein the cells into which the accessory molecule ligand gene is to be introduced are isolated from the animal or patient and then the gene is introduced into those isolated cells using suitable methods. Examples of useful _ex vivo_ methods have been described for example by Raper, S. E., M. Grossman, D. J. Rader, J. G. Thoene, B. J. Clark, D. M. Kolansky, D. W. Muller, and J. M. Wilson, _Ann. Surg._, 223:116 (1996); Lu, L., R. N. Shen, and H. E. Broxmeyer, _Crit. Rev. Oncol. Hematol._, 22:61 (1996); Koc, O. N., J. A. Allay, K. Lee, B. M. Davis, J. S. Reese, and S. L. Gerson, _Semin. Oncol._, 23:46 (1996); Fisher, L. J. and J. Ray, _Curr. Opin. Neurobiol._, 4:735 (1994); and, Goldspiel, B. R., L. Green, and K. A. Calis, _Clin. Pharm._, 12:488 (1993). D. Dilloo et al., in _Blood_ 90:1927-1933 (1997), describe a method, using CD40L-activated cells, for treating B-acute lymphoblastic leukemia (ALL). They cocultured leukemia cells with fibroblasts infected with a retroviral vector encoding CD40L, then injected the cell mix into mice. Such an approach, if taken in humans, would differ from that contemplated here in that the therapeutic cells are stimulated _in vitro_, by another cell line expressing the accessory molecule ligand. Schultze, J.L. et al., in _Blood_ 89: 3806-3816 (1997), describe a method for stimulating T-TILs (tumor-infiltrating T cells) cytotoxic for follicular lymphoma (FL) cells by exposing

them, *in vitro*, to FL B cells which were previously cultured with CD40L-expressing fibroblasts. They propose an adoptive immunotherapy in which T-TILS stimulated in this manner are transfused into patients. This method also requires *in vitro* stimulation, of the cells to be transfused, with another cell line expressing an accessory molecule.

Following the introduction of the gene, including any optional steps to assure that the accessory molecule ligand gene has been successfully introduced into those isolated cells, the isolated cells are introduced into the patient either at a specific site or directly into the circulation of the patient. In preferred embodiments of the present invention, cell surface markers, including molecules such tumor markers or antigens identify the cells are used to specifically isolate these molecules from the patient. One of ordinary skill in the art will understand that such isolation methods are well known and include such methodologies as fluorescence activated cell sorting (FACS), immunoselection involving a variety of formats including panning, columns and other similar methods.

The present invention also contemplates introducing the accessory molecule ligand gene into the desired cells within the body of an animal or human patient without first removing those cells from the patient. Methods for introducing genes into specific cells *in vivo*, or within the patient's body are well known and include use of gene therapy vectors and direct injection of various genetic constructs into the animal or patient. Examples of useful methods have been described by Danko, I. and J. A. Wolff, *Vaccine*, 12:1499 (1994); Raz, E., A. Watanabe, S. M. Baird, R. A. Eisenberg, T. B. Parr, M. Lotz, T. J. Kipps, and D. A. Carson, *Proc. Natl. Acad. Sci. U. S. A.*, 90:4523 (1993); Davis, H. L., R. G. Whalen, and B. A. Demeneix, *Hum. Gene Ther.*, 4:151 (1993); Sugaya, S., K. Fujita, A. Kikuchi, H. Ueda, K.

Takakuwa, S. Kodama, and K. Tanaka, Hum. Gene Ther., 7:223 (1996); Prentice, H., R. A. Kloner, Y. Li, L. Newman, and L. Kedes, J. Mol. Cell Cardiol., 28:133 (1996); Soubrane, C., R. Mouawad, O. Rixe, V. Calvez, A. Ghoumari, O. Verola, M. Weil, and D. Khayat, Eur. J. Cancer, 32A:691 (1996); Kass-Eisler, A., K. Li, and L. A. Leinwand, Ann. N. Y. Acad. Sci., 772:232 (1995); DeMatteo, R. P., S. E. Raper, M. Ahn, K. J. Fisher, C. Burke, A. Radu, G. Widera, B. R. Claytor, C. F. Barker, and J. F. Markmann, Ann. Surg., 222:229 (1995); Addison, C. L., T. Braciak, R. Ralston, W. J. Muller, J. Gauldie, and F. L. Graham, Proc. Natl. Acad. Sci. U. S. A., 92:8522 (1995); Hengge, U. R., P. S. Walker, and J. C. Vogel, J. Clin. Invest., 97:2911 (1996); Felgner, P. L., Y. J. Tsai, L. Sukhu, C. J. Wheeler, M. Manthorpe, J. Marshall, and S. H. Cheng, Ann. N. Y. Acad. Sci., 772:126 (1995); and, Furth, P. A., A. Shamay, and L. Hennighausen, Hybridoma, 14:149 (1995). In a typical application, a gene therapy vector containing an accessory molecule ligand gene is introduced into the circulation or at a localized site of the patient to allow the gene therapy vector to specifically infect the desired cells. In other preferred embodiments the gene therapy vector is injected directly into the tumor bed present in an animal which contains at least some of the cells into which the accessory molecule ligand gene is to be introduced.

The present invention also contemplates the direct injection of DNA from a genetic construct which has a promoter and accessory molecule ligand gene followed by a polyadenylation sequence into a patient or animal. Examples of such useful methods have been described by Vile, R. G. and I. R. Hart, Ann. Oncol., 5 Suppl 4:59 (1994). The genetic construct DNA is directly injected into the muscle or other sites of the animal or patient or directly into the tumor bed of the animal or patient. Alternatively, DNA from a genetic construct containing

at least an accessory molecule ligand gene is used and directly injected into the animal.

In preferred embodiments of the present invention, the immune reaction or response of a human patient or animal is altered by introducing the accessory molecule ligand gene into cells, including human cells which have an accessory molecule present on the cell surface. Such cells include human cells, human antigen presenting cells and optionally these cells may be neoplastic antigen presenting cells which have the capacity to express the accessory molecule on the surface of the cell or cells which are capable of stimulating. In some embodiments, the amount of accessory molecule present on the surface of the cells into which the accessory molecule ligand gene is to be introduced is very small and such small amounts of the accessory molecule may result from down-regulation of that accessory molecule on the surface of such cells. In some embodiments, the cells into which the accessory molecule ligand gene is introduced have at least low levels of the CD40 molecule present on the cell surface or are derived from cells which did express the CD40 ligand molecule on the cell surface but have reduced or eliminated that expression.

The preferred methods of altering the immunoreactivity of a particular cell are applicable to mammalian cells including human cells. These human cells may include neoplastic human cells such as human lymphomas, leukemias, and other malignancies including breast, lung and ovarian cancers. In some preferred embodiments the cells are normal antigen presenting cells of a human patient such as monocytes, macrophages, B cells, Langerhans cells, interdigitating cells, follicular dendritic cells, Kupffer cells, and other similar cells. In preferred embodiments, the cells are lymphocytes which acquire altered immunoreactivity when the accessory molecules of the present invention are introduced into those cells. In other preferred

embodiments, the cells may be neoplastic or normal cells which are capable of stimulating bystander antigen presenting cells when the accessory molecule ligand genes of the present invention are introduced into these cells. The present invention also contemplates that cells which are not naturally capable of presenting antigen to the immune system may be genetically engineered to introduce the genes encoding the molecules required for antigen presentation, including genes encoding an accessory molecule, and thus allow these cells to act as artificial antigen presenting cells. The accessory molecule ligand gene may then be introduced into these artificial antigen presenting cells. Various tests are well known in the literature to determine whether a particular cell is able to function as an antigen presenting cell, such as cell proliferation or the production of lymphokines and therefore this aspect of the present invention may be easily determined.

In addition to the above normal human cells, the present invention also contemplates introducing the accessory molecule ligand gene into various neoplastic or malignant cells which optionally are antigen presenting cells. Such human neoplastic cells which are contemplated include leukemias, lymphomas, AML, AMML, or CMML, CML, CLL and any neoplastic cell which is capable of stimulating bystander antigen presenting cells when an accessory molecule ligand is introduced into that cell. Also contemplated are neoplastic cells such as a breast, ovarian or lung cancer cell which is capable of or is engineered to act as an antigen presenting cell. However, the present immunomodulation also applicable to other malignancies not specifically identified and thus would include any tumor of any cell capable of presenting antigen within the animal or human immune system or any cell which is capable of acting as an antigen presenting cell or capable of stimulating

bystanding antigen presenting cells after an accessory molecule ligand gene has been introduced into those cells. Generally these antigen presenting cells have accessory molecules on the surface of the cells.

The present methods of altering the immunoreactivity of a human or animal cell contemplate the introduction of an accessory molecule ligand gene into the cells for which altered immunoreactivity is desired. The genes useful in the present invention include the wide range of accessory molecule ligand genes and chimeric accessory molecule ligand genes identified above and in preferred embodiments include at least a portion of the murine CD40 ligand gene. In particularly preferred embodiments, the accessory molecule ligand gene introduced into the cells using the methods of the present invention is selected to correspond to the accessory molecule present on the surface of the cells for which altered immunoreactivity is desired. In one particular application of the present invention, the immunoreactivity of a cell which expresses the CD40 molecule on the cell surface would be accomplished by introducing the gene which encodes the CD40 ligand molecule and more preferably the murine CD40 ligand molecule.

The present invention also contemplates altering the immunoreactivity of human or animal cells by introducing an accessory molecule ligand gene which is a chimeric accessory molecule ligand gene into the cell. The various useful chimeric accessory molecule ligand genes were identified above and could include a wide variety of molecules and allow the unique properties of those chimeric accessory molecule ligand genes to be utilized to alter the immunoreactivity of the target cells. In preferred embodiments, useful chimeric accessory molecule ligand genes are genes which encode at least a portion of the accessory molecule ligand which is capable of binding the accessory molecule

present on the surface of the cells for which altered immunoreactivity is desired.

The methods of the present invention for altering the immunoreactivity contemplate the use of genetic vectors and genetic constructs including gene therapy vectors which encode an accessory molecule ligand and therefore contain an accessory molecule ligand gene. Typically, the genetic vectors and genetic constructs including the gene therapy vectors of the present invention have a promoter which is operatively linked to the accessory molecule ligand gene followed by a polyadenylation sequence. In other embodiments, the only requirement is that the genetic vectors, genetic constructs, and gene therapy vectors of the present invention contain the accessory molecule ligand gene or the chimeric accessory molecule ligand gene.

V. Methods of Treating Neoplasia

The present invention also contemplates methods of treating human neoplasia comprising inserting into a human neoplastic cell a gene which encodes an accessory molecule ligand so that the accessory molecule ligand is expressed on the surface of the neoplastic cells. The present invention contemplates treating human neoplasia both in vivo, ex vivo and by directly injecting various DNA molecules containing a gene which encodes an accessory molecule ligand into the patient. However, at a minimum, the present methods for treating human neoplasia involve inserting the gene encoding the accessory molecule ligand into the neoplastic cells in such a way as to allow those neoplastic cells to express the accessory molecule ligand on the cell surface. The expression of the accessory molecule ligand gene in these neoplastic cells modulates the immune system to cause the neoplasia to be reduced or eliminated.

In a preferred method of treating human neoplasia, the method further comprises the steps of first

obtaining the human neoplastic cells from a human patient and then inserting into the isolated human neoplastic cells a gene which encodes an accessory molecule ligand so that the accessory molecule ligand is expressed on the surface of the neoplastic cells. The human neoplastic cells having the accessory molecule ligand on the surface of that cell are then infused back into the human patient. One of ordinary skill in the art will understand that numerous methods are applicable for infusing the altered human neoplastic cells containing the gene encoding the accessory molecule ligand back into the patient and that these methods are well known in the art.

The contemplated methods of treating human neoplasia are applicable to a wide variety of human neoplasias including lymphomas, leukemias, and other malignancies. In preferred embodiments the human neoplasia is a neoplasia which involves the antigen presenting cells of the human immune system and includes monocytes, macrophages, B cells, Langerhans cells, interdigitating cells, follicular dendritic cells, Kupffer cells, and the like. In other preferred embodiments, the human neoplasia is a leukemia, a lymphoma, AML, AMML, CMML, CML or CLL, lung cancer, breast cancer, ovarian cancer and other similar neoplasias.

The genetic vectors, genetic constructs and gene therapy vectors useful in the methods of treating human neoplasia of the present invention have been disclosed above and include constructs in which a promoter is operatively linked to the accessory molecule ligand gene or the chimeric accessory molecule ligand gene which is in turn operatively linked to a polyadenylation sequence. The methods of treating human neoplasia contemplate the use of genetic constructs, genetic vectors and gene therapy vectors as described in this specification. In addition, the present invention

contemplates the use of DNA which contains at least a gene encoding an accessory molecule ligand gene. This gene may or may not contain a promoter and other regulatory sequences.

In preferred embodiments of the present invention, the cells comprising the human neoplasia are located in at least one defined site termed a tumor bed within the human patient. This tumor bed typically contains the tumor or neoplastic cell together with a number of other cells which are associated with the tumor or neoplastic cells. The present invention contemplates methods of treating such human neoplasia present in a tumor bed by injecting into the tumor bed of the patient, a gene which encodes an accessory molecule ligand so that the accessory molecule ligand is expressed on the surface of the tumor cells thereby causing the cells to participate in an immune reaction. The gene which encodes the accessory molecule ligand may be present as part of a gene therapy vector, genetic construct or genetic vector.

In preferred embodiments, the accessory molecule ligand gene is a chimeric accessory molecule ligand gene which has at least a portion of the murine CD40 ligand gene is used. In other preferred embodiments, the accessory molecule ligand encoded is capable of binding an accessory molecule present on the human neoplasia to be treated.

The various gene therapy vectors used in the treatment methods of the present invention include vectors which are capable of directly infecting human cells. Such vectors have been described in the literature and are readily adaptable to the methods described in the present invention.

The present invention contemplates the use of any type of gene therapy including the methods of Raper, S.E. et al., Ann. Surg., 223:116 (1996); Lu, L. et al., Crit. Rev. Oncol. Hematol., 22:61 (1996); Koc, O. N. et

al., Semin. Oncol., 23:46 (1996); Fisher, L. J. et al., Curr. Opin. Neurobiol., 4:735 (1994); Goldspiel, B. R. et al., Clin. Pharm., 12:488 (1993); Danko, I. et al., Vaccine, 12:1499 (1994); Raz, E. et al., Proc. Natl. Acad. Sci. U.S.A., 90:4523 (1993); Davis, H. L. et al., Hum. Gene Ther., 4:151 (1993); Sugaya, S. et al., Hum. Gene Ther., 7:223 (1996); Prentice, H. et al., J. Mol. Cell Cardiol., 28:133 (1996); Soubrane, C. et al., Eur. J. Cancer, 32A:691 (1996); Kass-Eisler, A. et al., ann. N. Y. Acad. Sci., 772:232 (1995); DeMatteo, R. P. et al., Ann. Surg., 222:229 (1995); Addison, C. L. et al., Proc. Natl. Acad. Sci. U.S.A., 92:8522 (1995); Hengge, U. R. et al., J. Clin. Invest., 97:2911 (1996); Felgner, P. L. et al., Ann. N. Y. Acad. Sci., 772:126 (1995); Furth, P.A., Hybridoma, 14:149 (1995); Yovandich, J. et al., Hum. Gene Ther., 6:603 (1995); Evans, C.H. et al., Hum. Gene Ther., 7:1261.

VI. Methods of Vaccination

The present invention contemplates methods of vaccinating an animal against a predetermined organism comprising administering to that animal a vaccine containing immunogenic animal antigens capable of causing an immune response in that animal against the desired organism together with a vector containing a gene encoding an accessory molecule ligand. The present invention also contemplates methods of vaccinating an animal which include administering the genes which encode the immunogenic antigen capable of causing a desired immune response or altering the immune response to a particular antigen together with a vector containing a gene including the accessory molecule ligand gene. In this particular embodiment, the vector or vectors introduced encode the immunogenic antigens desired and the desired accessory molecule ligand. The present invention also contemplates that the gene or genes encoding the immunogenic peptide or peptides may

be present on the same vector as is the gene or genes encoding the accessory molecule ligand.

The vaccination methods of the present invention are general in that they may be used to produce a vaccination against any predetermined organism, such as a virus, a bacteria, a fungus or other organism. In addition, the present vaccination methods may be used to produce an immune response against a neoplastic cell.

In other preferred embodiments, the vaccination methods of the present invention utilize a genetic vector, a genetic construct or a gene therapy vector which contains an accessory molecule ligand gene which is a chimeric accessory molecule ligand gene. That chimeric accessory molecule ligand gene preferably contains at least a portion of the murine CD40 ligand gene. In other preferred embodiments, the vaccination method utilizes a DNA molecule which encodes at the minimum the accessory molecule ligand gene or a chimeric accessory molecule ligand gene. This particular DNA may or may not include a promoter sequence which directs the expression of the accessory molecule ligand gene.

The present invention also contemplates that the vaccination method may utilize a genetic vector which is capable of expressing an accessory molecule ligand within a particular cell or organism together with a vector which is capable of expressing at least a single polypeptide from an andovirus. This andovirus polypeptide may be expressed from the same or different vector which expresses the accessory molecule ligand in that cell. In this particular embodiment, the andovirus polypeptide is also expressed in at least one cell type within the organism and serves to modulate the immune response found in response to this vaccination protocol.

The present invention also contemplates the introduction of an accessory molecule ligand gene into cells which are present in the joints of patients with rheumatoid arthritis. In preferred embodiments, the

accessory molecule ligand gene introduced comprises at least a portion of the Fas ligand gene and upon expression the accessory ligand induces the cell death of cells expressing Fas on the cell surface. This process leads to the reduction of the destructive inflammatory process.

The following examples are provided to illustrate various aspects of the present invention and do not limit the scope of that invention.

VII. Methods of Treating Arthritis

The present invention also contemplates methods of treating arthritis comprising inserting into a joint, cells which have been transformed with an accessory molecule, such as the Fas ligand. In preferred embodiments, the expression of that accessory molecule ligand or the stability of that molecule on the surface of the cells has been altered. In these preferred embodiments, the accessory molecule ligand functions in an enhanced manner to aid in the treatment of arthritis within the joint. The present invention contemplates treating human arthritis both in vivo, ex vivo, and by directly injecting various DNA molecules containing genes which encode the useful accessory molecule ligand into the patients. Various useful protocols may be designed to rheumatoid arthritis including those described in the example section below.

The present invention contemplates the treatment of arthritis utilizing accessory molecule ligand genes which may be chimeric accessory molecule ligand genes comprised of portions of that gene being derived from two different accessory molecule ligand genes. In other embodiments, the chimeric accessory molecule ligands may be produced by utilizing domains from the same accessory molecule ligand gene. The resulting chimeric accessory molecule ligands have an altered stability on the surface of cells upon which they are expressed. This

altered stability modulates the function of the immune system in the local environment around the cells in which these chimeric accessory molecule ligands are expressed. For example, in certain preferred embodiments, Fas ligand stability is altered on the surface of cells within a joint of a patient suffering from arthritis. This altered stability modulates the immune system and causes the cells to be targeted for apoptosis and thus reducing the immune response within the inflamed joint. In other embodiments, the accessory molecule ligand genes described within are altered such that the resulting accessory molecule ligand has an altered stability and causes an immunomodulatory effect which can be useful in the treatment of arthritis.

The present invention contemplates in preferred embodiments that chimeric accessory molecule ligands genes be utilized in the treatment of arthritis. These chimeric accessory molecule ligand genes preferably contain at least a portion of the Fas ligand gene Domain IV, which carries the effect or function for Fas ligand. In preferred embodiments, at least in the portion of that domain, is present which allows Fas ligand to have its biologic effects. In other preferred chimeric accessory molecule ligands, those ligands contain domains from other accessory molecule ligand genes of the present invention or from a different domain of the same accessory molecule ligand. Particularly preferred are Fas chimeric accessory molecule ligand genes made up on Domain IV of the human Fas ligand operatively linked with Domain III of the mouse Fas ligand. This particular combination results in more stable Fas ligand and thus, by replacing Domain III of human Fas ligand with Domain III of the mouse ligand, the activity of the human Fas ligand gene is altered.

Alternatively, in other preferred embodiments, the murine Fas ligand gene is used to encode the murine Fas ligand on the surface of cells in place of the human Fas

ligand. The murine Fas ligand is more stable than the human Fas ligand and thus, alters the Fas ligand activity in the joint. The resulting alter Fas ligand activity is useful in the treatment of rheumatoid arthritis.

Further preferred embodiments include embodiments in which the effect or function present on Domain IV of the humand Fas ligand is combined with other domains from other accessory molecule ligands. For example, CD70 Domain III is more stable than Domain III of the human Fas ligand and thus the chimeric accessory molecule ligand made up of Domain III from the human CD70 and Domain IV of the Fas ligand together with other supporting domains would be more stable. The increased stability leads to increase Fas ligand activity. In other preferred embodiments, Domain III of the Fas ligand is replacd with multiple copies of a domain or domains. Such multiple copies of domains include domains made up of two or more copies of other domains such as Domains III or I of the CD70 molecule.

In other preferred embodiments, the present invention contemplates accessory molecule ligand genes, such as Fas ligand genes, in which a cleavage site for matrix-metalloproteinase (MMP), have been removed from the accessory molecule ligand. MMP cleavage and recognition sites, charted in Figure 28, are discussed in Smith, M.M. et al., Journal of Biol. Chem. 270:6440-6449 (95) and Nagase, H., and G.B. Fields, Biopolymers (Peptide Science) 40:399-416 (96). In preferred embodiments, at least one MMP site has been removed from at least Domain III of the Fas ligand gene. The removal of the MMP site from the Fas ligand gene makes the Fas ligand more stable and thus, more effective in the treatment of arthritis.

In other preferred embodiments, chimeric accessory molecule ligand genes are comprised of portions of the human Fas ligand gene with other domains from other

human accessory molecule ligands or domains from accessory molecules derived from other species. For example, the present invention contemplates the use of domains from CD40 ligand, CD70 ligand, CD30 ligand, TNF-related apoptosis inducing ligand (TRAIL), TNF-α as well as mutants of human Fas ligand and murine Fas ligand. Production of such chimeric accessory molecule ligands is easily accomplished by manipulating and producing accessory molecule ligand genes which are chimeric and thus has portions derived from at least two different accessory molecule ligand genes.

EXAMPLES

1. Expression of Human and Mouse Accessory Molecule Ligand in Human CLL Cells

a. Construction of a Genetic Construct and Gene Therapy Vector Containing a Human and Mouse Accessory Molecule Ligand Gene

Either the human accessory molecule ligand gene (human CD40 ligand) or the murine accessory molecule ligand gene (murine CD40 ligand) was constructed utilizing the respective human and murine genes. Each of these genes was cloned in the following manner.

i. Murine CD40-L cloning

Total RNA was isolated using the RNA STAT-60 kit (Tel-Test "B" Inc., Friendswood, TX) from 1 x 10^7 B6 mouse splenocytes that were previously activated for 8 hours with immobilized CD3-specific mAb. cDNA was then synthesized with the Superscript cDNA synthesis kit (Gibco BRL, Grand Island, NY) using oligo-dT primers. The murine CD40 ligand (mCD40-L) gene was then amplified from the cDNA by PCR using the following mCD40-L specific primers. 5'-GTTAAGCTTTTCAGTCAGCATGATAGAA (SEQ ID NO: 26), 5'-GTTTCTAGATCAGAGTTTGAGTAAGCC (SEQ ID NO: 27). The amplified mCD40-L PCR product was subcloned into the HindIII and XbaI sites of the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA). A DNA fragment encompassing the CMV promoter, mCD40-L gene, and polyadenylation signal was released from this plasmid construct after restriction digestion with BglII and XhoI enzymes. This DNA fragment was then subcloned into the shuttle plasmid MCS(SK)pXCX2 (Spessot R, 1989, Virology 168:378) that was designated mCD40-L pXCX2. This plasmid was used for adenovirus production as described below.

ii. Human CD40-L Cloning

A plasmid containing the gene for human CD40-L was used to produce the human CD40-L gene used herein. The

sequence of this gene is available and thus this source of the gene was used merely for convenience. See GenBank accession no. X67878. This plasmid was used for PCR amplification of the human CD40-L gene using the specific primers, sense primer 5' CCAAGACTAGTTAACACAGCATGATCGAAA 3' (SEQ ID NO: 28) and antisense primer 5' CCAATGCGGCCGCACTCAGAATTCAACCTG 3' (SEQ ID NO: 29).

These primers contain flanking restriction enzyme sites for subcloning into the eukaryotic expression plasmid pRc/CMV (Invitrogen). The PCR amplified CD40-L fragment was subcloned into the SpeI and NotI sites of pRc/CMV and designated hCD40-L pRc/CMV. A BglII and XhoI fragment encompassing the CMV promoter, hCD40-L gene, and polyadenylation signal was then released from this plasmid and subcloned into the shuttle plasmid MCS(SK)pXCX2 as described above. This plasmid was designated hCD40-L pXCX2. This plasmid was used for adenovirus production as described below.

iii. Adenovirus Synthesis

Either mCD40-L pXCX2 or hCD40-L pXCX2 plasmids were co-transfected with pJM17 (Graham and Prevec, 1991, Methods in Molecular Biology, Vol 7) into 293 cells (American Type Culture Collection, Rockville, MD) using the calcium phosphate method (Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning, A Laboratory Manual, 2nd edition, chapter 16:33-34). Isolated adenovirus plaques were picked and expanded by again infecting 293 cells. High titer adenovirus preparations were obtained as described (Graham and Prevec, 1991, Methods in Molecular Biology, Vol 7), except for the following modifications. The cesium chloride gradient used for concentrating viral particles was a step gradient, with densities of 1.45 g/cm^3 and 1.2 g/cm^3. The samples were spun in a SW41 rotor (Beckman, Brea, CA) at 25,000 rpm at 4°C. The viral band was desalted using a Sephadex

G25 DNA grade column (Pharmacia, Piscataway, NJ). The isolated virus was stored at 70°C in phosphate buffered saline with 10% glycerol. The virus titer was determined by infecting 293 cells with serial dilutions of the purified adenovirus and counting the number of plaques formed. Viral titers typically ranged from 10^{10} to 10^{12} plaque forming units/ml (PFU/ml).

b. Introduction of a Murine and Human Accessory Molecule Ligand Gene into CLL Cells and HeLa Cells

For adenovirus infection, 10^6 freshly thawed and washed CLL cells or HeLa cells were suspended in 0.5 to 1 mL of culture medium for culture at 37°C in a 5% CO_2-in-air incubator. Adenovirus was added to the cells at varying multiplicity of infection (MOI), and the infected cells were cultured for 48 hours, unless otherwise stated, before being analyzed for transgene expression.

c. Expression of an Accessory Molecule Ligand Gene in CLL Cells and HeLa Cells

The CLL and HeLa cells which were infected with the adenovirus vector containing either mouse or human CD40 ligand genes prepared in Example 1b. were then stained with commercially available monoclonal antibodies immunospecific for either human or mouse CD40 ligand (Pharmingen, San Diego, CA) using the manufacturer's directions. The CLL and HeLa cells were washed in staining media (SM) consisting of RPMI-1640, 3% fetal calf serum and 0.05% sodium azide and containing propidium iodide and then analyzed on a FACScan (Becton Dickinson, San Jose, CA). Dead cells and debris were excluded from analysis by characteristic forward and side light scatter profiles and propidium iodide staining. Surface antigen expression was measured as the mean fluorescence intensity ratio (MFIR). MFIR

equals the mean fluorescence intensity (MFI) of cells stained with a specific FITC-conjugated MoAb, divided by the MFI of cells stained with a control IgG-FITC. This method controls for the nonspecific increases in auto-fluorescence seen in larger, more activated cells.

The histograms, generated for the CLL cells and HeLa cells containing either a genetic vector containing the human CD40 ligand gene or the murine CD40 ligand gene and the appropriate controls, are shown in Figure 3A-3D. The expression of both the murine and human accessory molecule ligand gene (CD40 ligand) in HeLa cells is shown in Figures 3A and 3B, respectively. The expression of the murine and human accessory molecule ligand in CLL cells is shown in Figures 3C and 3D. The expression of an accessory molecule ligand gene in CLL cells and the expression of murine CD40 ligand on the surface of the CLL cells is shown in Figure 3C. The failure of the human accessory molecule ligand to be expressed on the surface of the CLL cells is shown in Figure 3D.

Figure 8 shows data from an experiment done to examine whether the $CD4^+$ T cells of CLL patients could be induced to express the accessory molecule ligand mRNA after CD3 ligation. An ELISA-based quantitative competitive RT-PCR was used to measure CD40 ligand transcript levels. In this experiment, CD40 ligand and RNA transcribed from the CD40 ligand gene in CLL cells are compared with levels of CD40 ligand and RNA made in normal donor cells, after induction by CD3 ligation. For CD3 activation, plate coats of CD3 mAb were made and incubated with plated CLL or normal donor mononuclear cells for the indicated amount of time, after which cells were analyzed for expression of surface antigens or CD154 RNA message levels. CLL or normal donor serum was added to the cells at the beginning of the activation assay for examination of modulation of CD40 ligand surface expression.

For quantitative CD154 RT-PCR ELISA, total RNA was extracted and competitor RNA was generated from the insert containing CD40 ligand (CD154) cDNA. Varying amounts of competitor RNA were added to separate wells of isolated total RNA that subsequently were converted into cDNA. CD3 activation, ELISAs and PCR reactions were performed as described in Cantwell, M. et al., Nature Medicine 3:984-989 (1997). Biotinylated PCR products were captured onto microtiter plates (Becton Dickinson, Oxnard, CA) coated with streptavidin (Sigma), and incubated. The plate was treated with NaOH to remove the sense strands and subsequently washed. The DNA was then hybridized with either wild-type gene-specific or competitor-specific oligonucleotides. Using terminal transferase, each probe was labeled with a molecule of digoxigenin-11-dideoxyUTP (Boehringer Mannheim). The plate was incubated and washed with HYBE buffer and blocking buffer, then peroxidase-conjugated anti-digoxigenin antibody (150 U/ml; Boehringer Mannheim) in blocking buffer was added. TMB (tetramethylbenzidine) and peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were added for color development, and optical densities were measured at 450 nm and Deltasoft II (Biometallics, Princeton, NJ) was used for data analysis.

Standard curves plotting the moles of RNA product versus the optical density were made for the standard cDNA reactions. The equations describing these standard curves were then used to calculate the moles of wild-type or competitor DNA present in the unknown PCR reactions based on the optical densities obtained in the ELISA readings. The ratio of the quantity of wild-type DNA to the amount of competitor DNA was then plotted against the known quantity of competitor RNA added in the initial samples. The ratio of 1 was taken for the extrapolation of the amount of unknown moles of target RNA in the sample (a ratio of 1 means the amount of

target RNA versus competitor RNA are equal). The molecules of target RNA per CD4 cell was then calculated based on the following formula: [(moles target CD154 RNA) x (6 X 10^{23} molecules/mole) x (dilution factor of test RNA)]/(% of CD4 T cells in total cell population).

The upper graph in Figure 8 shows that T cells of patients with CLL do not express detectable CD40 ligand after CD3 ligation. CD40 ligand RNA is produced, but it is not stable. Although both CD40 ligand and CD40 ligand RNA are expressed in normal donor T cells (lower graph), the levels of neither the protein or RNA are stably maintained.

Figure 9 shows a time course for surface expression of CD40 ligand. Expression reached a peak level at 48 hours after infection and persisted at high levels for at least 6 days thereafter. In this experiment, CLL B cells were infected with a gene therapy vector containing an accessory molecule ligand, at a MOI of 1000 at time zero, and then assessed by flow cytometry at various times thereafter. At each time point listed on the abscissa, the proportions of viable CLL B cells that expressed detectable CD154 are indicated by the vertical bars corresponding to the percentage scale depicted on the right-hand ordinate.

d. <u>Function of the Human and Murine Accessory Molecule Ligands</u>

i. <u>Induction of CD80 and CD54 on Cells Containing a Gene Therapy Vector Encoding an Accessory Molecule</u>

The CLL cells infected with the murine accessory molecule ligand gene prepared in Example 1b. were then cultured in tissue culture plates. The CLL cells were then analyzed using multiparameter FACS analysis to detect induction of CD80 and CD54 expression using fluroescein isothiocyanate-conjugated monoclonal antibodies immunospecific for each of these respective

surface antigens. Non-infected CLL cells were used as a control. The cells were subjected to the appropriate FACS analysis and histograms were generated. CD80 mAb was obtained from Dr. Edward Clark and CD54 mAb was purchased from CALTAG Inc. The CD80 was conjugated using standard methods which have been described in Kipps et al., Laboratory Immunology II, 12:237-275 (1992).

The results of this analysis are shown in Figure 4A-4D. Figures 4A-4B compare the amount of CD54 expression in CLL cells which have not been transfected (Figure 4A) or CLL cells into which a gene therapy vector containing the murine CD40 ligand gene was introduced (Figure 4B). The shaded graph indicates the isotype control for FACS staining and the open graph indicates the cells stained with the anti-CD54 antibody. These results show that the level of expression of CD54 is increased in CLL cells into which the gene therapy vector containing the murine CD40 ligand was introduced.

Figures 4C and 4D compare the amount of CD80 expression in CLL cells which have not been transfected (Figure 4C) or CLL cells into which a gene therapy vector containing the murine CD40 ligand gene was introduced (Figure 4D). The shaded graph indicates the isotype control for FACS staining and the open graph indicates the cells stained with the anti-CD80 antibody. These results show that the level of expression of CD80 is increased in the CLL cells into which the gene therapy vector containing the murine CD40 ligand was introduced.

In an additional experiment, CLL cells infected with a gene therapy vector containing the murine accessory molecule ligand gene were evaluated by flow cytometry for induced expression of not only CD54 and CD80, but also CD86, CD58, CD70 and CD95. Fluorescein-conjugated mAb specific for human CD54 and CD70 were purchased from CALTAG. Fluorescein-conjugated mAb

specific for human CD27, CD58, CD80, CD86, or CD95, and phycoerythrin-conjugated mAb specific for human or mouse CD40 ligand, were obtained from PharMingen. Shaded histograms represent staining of CLL B cells with FITC-conjugated isotype nonspecific mAb. In contrast to uninfected CLL cells (Figure 10, thin-lined histograms), or Ad-lacZ-infected CLL cells (data similar to that obtained with uninfected cells, but not shown), CLL cells infected with the adenovirus vector encoding the CD40 ligand (CD154) expressed high levels of CD54 (Figure 10, top left), CD80 (Figure 10, top middle), CD86 (Figure 10, top right), CD58 (Figure 10, bottom left), CD70 (Figure 10, bottom middle), and CD95 (Figure 10, bottom right). On the other hand, CD40 ligand-CLL (CD154 CLL) expressed significantly lower levels of both surface membrane CD27 (Figure 11A, thick-lined histogram) and soluble CD27 (Figure 11B) than uninfected (Figure 11A, thin-lined histogram)($P < 0.01$, Bonferroni t-test) or Ad-lacZ-infected CLL cells (data similar to that obtained with uninfected cells, but not shown). In the experiment shown in Figure 11A, the CLL B cells were examined for expression of CD27 via flow cytometry, three days after infection. Shaded histograms represent staining of CLL B cells with FITC-conjugated isotype control mAb. In Figure 11B, cell-free supernatants were collected, after the infection or stimulation of CLL B cells, for 72 hours and tested for the concentration of human CD27 by ELISA. The reduced expression of CD27 (Figure 11B) is similar to that noted for leukemia B cells stimulated via CD40 cross-linking with mAb G28-5 presented by CD32-expressing L cells, as described in Rassenti, L.Z. and T.J. Kipps, J. Exp. Med. 185:1435-1445.

ii. Allogeneic T Cell Responses to CLL Cells Into Which a Genetic Therapy Vector Containing a Murine CD40 Ligand Gene Has Been Introduced

The ability of CLL cells which have been infected with a gene therapy vector containing the murine CD40 ligand gene to stimulate allogeneic T cells (i.e., from another individual) was analyzed using cell proliferation assays. Briefly, the test cells were co-cultured with the genetic therapy vector containing the lac-Z gene or the murine CD40 ligand gene at a multiplicity of infection of 1,000 in the presence of IL-4 at a concentration of 10 ng/ml. In other samples, the CLL cells were stimulated with MOPC21 (a control IgG) or G28-5 (an anti-CD40 monoclonal antibody) or were preincubated on CD32-L cells and at the same time treated with IL-4. The preincubation with the CD32-L cells together with IL-4 treatment have been shown to be an efficient form of cross-linking the CD40 molecule other than direct gene transfection.

After three days of culture at 37°C, these cells were treated with mitomycin C to prevent their proliferation and then used to stimulate allogeneic T cells. Prior to this co-culture, the different aliquots of CLL cells had either been treated with the anti-CD40 monoclonal antibody or had been infected with the gene therapy vector containing either the lac-Z or murine CD40 ligand gene at a stimulator ratio of 1:10. After two days of culture at 37°C, interferon gamma (IFNg) production was measured by ELISA assay. After five days of co-culture at 37°C, the incorporation of ^{3}H-thymidine into replicating cells was measured after an eight hour pulse label. The results of this assay are shown in Table II below and in Figure 5.

In another experiment, CLL B cells infected with the gene therapy vector containing the CD40 ligand gene were evaluated for their ability to act as stimulator

cells in an allogeneic mixed lymphocyte T cell reaction (MLTR). In parallel, the stimulatory capacity of control lac-Z-vector-infected CLL cells and CLL B cells that had been cultured with CD32-L cells and an anti-CD40 mAb (G28-5) or an isotype control Ig, was also examined as described in Ranheim, E.A. and T.J. Kipps, J. Exp. Med., 177:925-935 (1993), Clark, E.A. and J.A. Ledbetter, Proc. Natl. Acad. Sci. USA, 83:4494-4498 (1986), and Banchereau, J. et al., Science 251:70-72 (1991). Effector T cells from a non-related donor were co-cultured with the CLL stimulator cells at an effector to target ratio of 4:1. After 18 h culture at 37°C, over 30% of the allogeneic $CD3^+$ cells were found to express the activation-associated antigen CD69 when cultured with CD154-CLL cells (data not shown). In contrast, less than 4% of the T cells expressed CD69 when co-cultured with uninfected or Ad-lacZ-infected CLL cells (data not shown).

Two days after the initiation of the MLTR, the concentrations of IFNg in the culture supernatants were assayed by ELISA. The supernatants of the MLTR stimulated with CLL cells infected with the accessory molecule ligand CD40L (Figure 12A, CD154-CLL) contained significantly higher levels of IFNg (306 ± 5 ng/ml, m ± SE, n = 3) than that of MLTR cultures stimulated with the anti-CD40 mAb (Figure 12A, aCD40-CLL) (23 ± 3 ng/ml) ($P < 0.05$, Bonferroni t-test). The latter was not significantly different from that of MLTR cultures stimulated with control Ad-lacZ-infected CLL cells (Figure 12A, lacZ-CLL) (43 ± 10 ng/ml) ($P > 0.1$, Bonferroni t-test). The supernatants of effector cells alone, or of MLTR cultures stimulated with uninfected CLL cells (Figure 12A, CLL) or control Ig treated CLL cells (Figure 12A, MOPC-CLL), did not contain detectable amounts of IFNg(<2 ng/ml). Similarly, none of the leukemia B cell populations produced detectable amounts

of IFNg when cultured alone, without added effector T cells (data not shown).

After 5 days, cell proliferation was assessed by incorporation of ^{3}H-thymidine. Cultures with isotype control IgG-treated (Figure 12B, MOPC-CLL) or uninfected (Figure 12B, CLL) stimulator cells did not incorporate more ^{3}H-thymidine than cultures without added leukemia-stimulator cells (Figure 12B, None). Ad-lacZ-infected CLL B cells (Figure 12B, lacZ-CLL) also were unable to stimulate allogeneic T cells to incorporate amounts of ^{3}H-thymidine that were much greater than that of control cultures. In contrast, anti-CD40-stimulated leukemia cells or CD154-CLL cells each induced significant effector cell proliferation (Figure 12B, aCD40-CLL or CD154-CLL) ($P < 0.05$, Bonferroni t-test). Moreover, the amount of ^{3}H-thymidine incorporated by cultures stimulated with CD154-CLL cells (41,004 ± 761 cpm (m ± SE), n = 3) was significantly greater than that of cultures stimulated with equal numbers of aCD40-CLL cells (22,935 ± 1,892 cpm, n = 3) ($P < 0.05$, Bonferroni t test). However, neither of these mitomycin-C-treated leukemia cell populations incorporated ^{3}H-thymidine when cultured without effector T cells (data not shown). Also, as described for the MLTR between allogeneic T cells and CD40-stimulated CLL cells {6549, 7167, 7168}, allogeneic T cell proliferation in response to CD154-CLL could be inhibited by CTLA-4-Ig or CD11a mAb when added at the initiation of the MLTR, indicating that respective interactions between CD80/CD86 and CD28, or CD54 and CD11a/CD18, contribute to the noted allogeneic T cell reaction (data not shown).

Table II

Allogeneic T cell responses to CLL cells infected with mCD40-L adenovirus

	% positive cells		Allogeneic response (mean±SEM)	
Stimulators	mCD40-L	Human CD80	3H-TdR uptake (cpm)	IFNγ production (ng/ml)
None (t cells only)	-	-	3577 ± 821	n.d.*
CLL with:				
No activation	0	1.4	4577 ± 1097	n.d.
MOPC21	0	1.0	5259 ± 1788	n.d.
G28-5	0	26.7	22935 ± 1892	22.3 ± 1.6
lac-Z adeno	0	4.8	9037 ± 1781	43.2 ± 10.5
mCD40-L adeno	17.5	19.7	41004 ± 761	305.7 ± 4.5

* n.d. - not detectable

iii. Stimulation of Gamma Interferon by CLL Cells Containing an Accessory Molecule Ligand Gene

The function of CLL cells containing an accessory molecule ligand gene (mouse CD40 ligand) was analyzed by determining the ability of those cells to activate T lymphocytes. The procedure was performed as follows: allogeneic T lymphocytes from a healthy donor (greater than 90% $CD3^+$) were purified using magnetic beads and monoclonal antibodies specific for the CD14 and CD19 antigen. These allogeneic T lymphocytes then were cultured together with MMC-treated CLL cells which were infected with the accessory molecule ligand gene (murine CD40 ligand) or the lac-Z gene. This co-culture was performed in RPMI-1640 medium containing 10% fetal calf

serum. After culture for 24 hours, the cells were collected and analyzed to determine the expression of the antigen CD69 on the T lymphocytes using a standard FACS sorting protocol. The cell culture supernatants were collected after two days in culture and tested to determine the concentration of human interferon gamma using an ELISA assay. A portion of the CLL cells containing an accessory molecule ligand gene (murine CD40 ligand) and a portion of the cells containing the adenovirus expressing the lac-Z were cultured in the presence of human interleukin 4 IL-4 (5 ng/mL). The production of interferon gamma by allogeneic T lymphocytes in the presence of this amount of human interleukin 4 was also analyzed. The results from these analyses are shown in Figure 6.

As can be seen, the human CLL cells containing the accessory molecule ligand gene (murine CD40) produced substantially higher concentrations of interferon gamma in the cell culture supernatant when compared to CLL cells which contained the lac-Z gene. The increased production of interferon gamma (IFNg) by T lymphocytes exposed to CLL cells containing the accessory molecule ligand gene indicates that these CLL cells containing the accessory molecule ligand genes were effective in producing an enhanced immune response.

iv. <u>Stimulation of Allogeneic T Cells Pre-Exposed to Non-Modified CLL B Cells Containing an Accessory Molecule Ligand Gene</u>

Prior studies indicated that antigen presentation to T cells, in the absence of the signals derived from costimulatory molecules such as CD28, can lead to specific T cell clonal anergy. For this reason, allogeneic T cells that had previously been cultured, with non-modified CLL B cells lacking expression of CD80 and other immune accessory molecules, were tested for

their ability to respond to CLL cells containing the CD40 ligand gene. Allogeneic effector cells did not incorporate more ^{3}H-thymidine in response to non-modified CLL cells (Figure 12C, CLL), or control CLL cells infected with Ad-lacZ (Figure 12C, lacZ-CLL), than when they were cultured alone (Figure 12C, None). In contrast, even after prior co-culture with non-modified CLL B cells, allogeneic effector cells could still be induced to proliferate (Figure 12C, CD154-CLL) or to produce IFNg (Figure 12D, CD154 CLL) in response to cells expressing an accessory molecule ligand. Although modest amounts of IFNg were detected in the supernatants of such secondary cultures when Ad-lacZ-infected leukemia cells were used as stimulator cells (Figure 12D, lacZ-CLL), this level was significantly lower than that noted for secondary cultures with Ad-CD40-ligand-infected CLL cells (Figure 12D, CD154-CLL) ($P < 0.05$, Bonferroni t-test). Similarly, the supernatants of the leukemia cells alone (data not shown), and the effector cells alone (Figure 12D, None), of the MLTR cultures stimulated with uninfected CLL cells (Figure 12D, CLL), contained negligible amounts of IFNg(<2 ng/ml). These results indicate that allogeneic effector cells cultured with nonmodified CLL B cells are not precluded from responding to CLL B cells infected with a gene therapy vector containing the accessory molecule ligand gene.

v. Autologous T Cell Responses to CLL Cells Into Which a Gene Therapy Vector Encoding a Murine Accessory Molecule Ligand Gene Has Been Introduced

T cells isolated from the blood of CLL patients were examined for their ability to respond in vitro to autologous CLL B cells containing a gene therapy vector which encodes the murine accessory molecule, CD40 ligand. T cells were isolated to >95% purity, and then co-cultured with mitomycin-C-treated autologous leukemia

cells in serum-free AIM-V medium supplemented with exogenous interleukin-2 at 25 U/ml. Modest ^{3}H-thymidine incorporation (≤10,000 cpm) was detected in cultures without added stimulator cells, secondary in part to the exogenous IL-2 (Figure 13A, and data not shown). The level of T cell proliferation, however, did not increase in response to uninfected CLL cells (Figure 13A, CLL) or Ad-lacZ-infected CLL cells (Figure 13A, lacZ-CLL). In contrast, CLL cells infected with a gene therapy vector containing the accessory molecule ligand (Figure 13A, CD154-CLL) induced autologous T cells to incorporate significantly more ^{3}H-thymidine (17, 368 ± 1,093 cpm, n=3) than any of the control cultures ($P < 0.05$, Bonferroni t-test). Furthermore, the MLTR stimulated with CLL cells infected with a vector encoding an accessory molecule ligand (CD40L) also generated significantly more IFNg (165 ± 3 ng/ml, n=3) than any of the other cultures (Figure 13B)($P < 0.05$, Bonferroni t-test).

The T cells were harvested after 5 days from the autologous MLTR and assessed for CTL activity against autologous CLL B cells. T cells co-cultured with autologous CD40-ligand-CLL cells developed CTL activity for non-modified CLL B cells, effecting 40.1% lysis (± 2.3%) at an E:T ratio of 2:1 (Figure 13C, CD154). However, such T cells did not develop detectable CTL activity for the same target cells in the control reactions, when co-cultured with uninfected or Ad-lacZ-infected CLL cells (Figure 13C).

vi. <u>Specificity of CTL Stimulated by Autologous CD40-Ligand-CLL B Cells for Allogeneic CLL B Cells</u>

Effector cells stimulated with autologous CD40-ligand-CLL were evaluated for their ability to secrete IFNg or manifest CTL activity against allogeneic CLL B cells (Figure 14). After 5 days of autologous MLTR with

CD154-CLL or lacZ-CLL, T cells were isolated by Ficoll density gradient centrifugation, washed extensively, and then cultured in media for 24 h. Washed T cells were mixed with autologous ("Auto CLL", solid bar) or allogeneic ("Allo-1 CLL" or "Allo-2 CLL", shaded or hatched bars) target CLL B cells. T cells stimulated in the autologous MLTR with CD40-ligand-CLL cells, but not with lacZ-CLL cells, produced significantly more IFNg in response to secondary culture with non-modified autologous CLL B cells than with allogeneic CLL B cells (Figure 14A)($P < 0.05$, Bonferroni t-test). Furthermore, T cells stimulated with CD40-ligand-CLL cells, but not with lacZ-CLL cells, were cytotoxic for autologous CLL cells, but not allogeneic CLL cells (Figure 14B). Similar results were obtained with the autologous MLTR-activated T cells of the allogeneic donor, again demonstrating specific cytotoxicity for autologous CLL B cells (data not shown). Finally, W6/32, a mAb to class I major histocompatibility complex (MHC I) antigens could significantly inhibit the cytotoxicity of T cells stimulated with CD40-ligand-CLL cells for autologous CLL B cells (Figure 14C, αHLA-class I))($P < 0.05$, Bonferroni t-test). Such inhibition was not observed with mAb specific for MHC class II antigen (Figure 14C, αHLA-DP), mAb specific for the Fas-ligand (Figure 14C, αFasL), or an isotype control mAb of irrelevant specificity (Figure 14C, MOPC-21). Collectively, these studies indicate that Ad-CD40-ligand-infected CLL cells can induce an autologous anti-leukemia cellular immune response <u>in vitro</u>, leading to the generation of MHC-class I-restricted CTL specific for autologous non-modified leukemia B cells.

e. <u>Transactivation of Non-Infected Bystander Leukemia B Cells by Ad-CD40L CLL Cells</u>

To address whether the changes in tumor marker expression (described in section 1di.) resulted from

intracellular versus intercellular stimulation, the effect of culture density on the induced expression of CD54 and CD80 following infection with adenovirus gene therapy vector encoding the accessory molecule ligand (CD40L, or CD154) was examined. After infection, CLL cells were cultured at standard high density (e.g. 1 x 10^6 cells/ml) or low density (e.g. 2 x 10^5 cells/ml) for 3 days at 37° C. Cells plated at high density contained homotypic aggregates, whereas cells plated at low density remained evenly dispersed and without substantial cell-cell contact (data not shown). Despite expressing similar levels of heterologous CD154, CD154-CLL B cells cultured at high density were induced to express higher levels of CD54 and CD80 than CD154-CLL cells cultured at low density (Figure 15A). The stimulation achieved at high density could be inhibited by culturing the cells with a hamster anti-mouse CD154 mAb capable of blocking CD40<->CD154 interactions (Figure 15B, aCD154 Ab). Collectively, these studies indicate that CD154-CLL cells can activate each other in *trans* and that surface expression of CD154 is necessary for optimal leukemia cell stimulation.

In addition, Ad-CD154-infected, uninfected, Ad-lacZ-infected, or G28-5-stimulated CLL cells were labeled with a green-fluorescence dye to examine whether CD154-CLL could stimulate non-infected bystander leukemia cells. Dye-labeled cells were used as stimulator cells for equal numbers of non-labeled syngeneic CLL B cells. After 2 days' culture, stimulator cells cultured by themselves retained the green-fluorescence dye, allowing such cells to be distinguished from non-labeled CLL cells by flow cytometry. Bystander (green-fluorescence-negative) $CD19^+$ CLL B cells were induced to express CD54 (Figure 15C, right histogram) or CD86 (Figure 15D, right histogram) when co-cultured with Ad-CD154-infected leukemia B cells, but not with mock infected CLL cells (Figures 15C

and 15D, left histograms), G28-5-stimulated CLL cells, or Ad-lacZ-infected CLL cells (data not shown). As expected, these bystander (green-fluorescence-negative) CLL cells also were negative for heterologous CD154.

f. Treatment of Leukemia with Gene Therapy Vectors Encoding an Accessory Molecule Ligand

Figure 24 shows an outline for a clinical trial for testing treatment of B cell CLL with adenovirus gene therapy vectors encoding modified CD40 ligand. Leukemia cells harvested by pheresis are infected with replication-defective vectors that encode the modified CD40 ligand. Following expression of this protein, the cells will be administered back to the patient for the purpose of stimulating a host anti-leukemia-cell immune response. This strategy is far superior to one that uses gene therapy to affect expression of only one immune stimulatory molecule on the leukemia cell surface. Indded, this strategy results in the leukemia cells expressing an array of immune-stimulatory accessory molecules and cytokines, as well as a molecule that can affect the same changes in leukemia cells of the patient that were never harvested.

2. Expression of Chimeric Accessory Molecule Ligand Genes

The chimeric accessory molecule ligand genes described below are prepared using standard techniques as described herein.

a. Preparation of Chimeric Accessory Molecule Ligand Genes Utilizing Domains from Two Different Accessory Molecule Genes

The human CD40 ligand gene was isolated from RNA prepared from T cells which had been activated by an anti-CD3 monoclonal antibody using 5′ and 3′ primers together with well known PCR methods. Chimeric accessory molecule genes of human CD40 ligand and murine

CD40 ligand are constructed from the newly cloned human CD40 ligand gene and mouse CD40 ligand gene described herein as SEQ ID NO: 2., The transmembrane and cytoplasmic domains of human CD40 ligand genes are exchanged with those of the murine CD40 ligand gene and designated H(Ex)-M(Tm-Cy) CD40 ligand. These chimeric accessory molecule ligand genes are produced using the gene conversion technique described as SOEN which has been previously described by Horton, Mol. Biotechnol., 3:93 (1995). A diagram depicting the chimeric accessory molecule ligand genes which are produced is shown in Figure 4. The nucleotide sequences of each of these respective chimeric accessory molecule ligand genes is designated SEQ ID NOS: 3-7 as indicated in the Table below.

Table III

Chimeric Accessory Molecule Ligand Gene	SEQ ID NO:
HuIC/HuTM/MuEX CD40-Ligand	SEQ ID NO: 3
HuIC/MuTM/HuEX CD40-Ligand	SEQ ID NO: 4
HuIC/MuTM/MuEX CD40-Ligand	SEQ ID NO: 5
MuIC/HuTM/HuEX CD40-Ligand	SEQ ID NO: 6
MuIC/MuTM/HuEX CD40-Ligand	SEQ ID NO: 7

Adenovirus vectors encoding each of the chimeric accessory molecules shown in Figure 2 are constructed using the methods described in Example 1. Each of these constructs are then transfected into either HeLa cells or CLL cells according to the methods of Example 1.

b. Expression of Chimeric Accessory Molecule Ligands on CLL and HeLa Cells

The expression of each of the chimeric accessory molecule ligand genes constructed above is analyzed by using FACS analysis as specified in Example 1. The appropriate monoclonal antibody immunospecific for the external domain of either human or mouse CD40 ligand is selected and used to determine the level of expression

of the chimeric accessory molecules on the surface of these cells. After appropriate analysis and preparation of appropriate histograms, the expression of chimeric accessory molecules containing at least a portion of the murine CD40 ligand gene is confirmed.

c. Function of Chimeric Accessory Molecule Ligands

CLL cells are infected with various MOI of the mCD40L adenovirus and then cultured in 48 or 24 well tissue culture plates for various times after infection (48, 72, and 96 hours). The $CD19^{+}$ B cells are then analyzed by multiparameter FACS analysis for induction of CD80 and CD54 expression using fluroescein isothiocyanate-conjugated mAb specific for each respective surface antigen as described in Example 1. Increased amounts of CD54 and CD80 are found on cells which have the chimeric accessory molecules containing the domain or domains derived from the mouse CD40 ligand gene.

Further analysis of the cells containing the chimeric accessory molecule genes is carried out according to Example 1(d). The cells containing the chimeric accessory molecule genes which contain the domains derived from the murine CD40 ligand gene are able to stimulate the production of gamma interferon and T cell proliferation.

d. Expression of Chimeric Accessory Molecule Genes Which Contain Proximal Extracellular Domains from Two Different Accessory Molecules from the Same Species

A chimeric accessory molecule ligand gene is prepared which contains the proximal extracellular domain from the human CD70 gene (Domain III) with the remainder of the domains derived from the human CD40 ligand gene. This gene is prepared using standard

biologic techniques as previously described herein. This chimeric accessory molecule ligand gene has the DNA sequence shown as SEQ ID NO: 19. A different chimeric accessory molecule ligand gene is prepared which contains the proximal extracellular domain from the murine CD40 ligand gene with the remainder of the domains derived from the human CD40 ligand gene. This gene is prepared using standard techniques as previously described herein. This chimeric accessory molecule ligand gene has the DNA sequence shown as SEQ ID NO: 20.

The chimeric accessory molecule genes shown as SEQ ID NOS: 19 and 20 are inserted into the appropriate vectors as described in Example 1 and introduced into human neoplastic cells. The expression of that chimeric accessory molecule gene in the cells is determined as was described in Example 1.

The chimeric accessory molecule encoded by each of these chimeric accessory molecule genes is found on the surface of the human neoplastic cells using the FACS analysis described in Example 1. Increased amounts of CD54 and CD80 are found on the cells containing the chimeric accessory molecule genes using the techniques described in Example 1. The cells containing the chimeric accessory molecule gene are able to stimulate the production of gamma interferon and T cell proliferation as described and assayed according to Example 1.

3. <u>Augmentation of Vaccination Using Vectors Encoding Accessory Molecules</u>

The following procedures were used to demonstrate the augmentation of a vaccination protocol using a gene therapy vector encoding an accessory molecule.

a. <u>Augmentation of the Antibody Response in Mice Co-Injected with an Accessory Molecule Gene Therapy Vector and placZ</u>

Three different gene therapy constructs were prepared using standard techniques including those techniques described herein. The first was a control gene therapy vector, pcDNA3, which did not contain any gene. The second, placZ, contained the Lac-Z gene which encoded β-galactosidase (β-gal). The third, p-mCD40L, contained the murine CD40 ligand gene described in Example 1.

Prior to any immunizations, serum was isolated from 6-8 week old BALB/c-mice to determine the amount of any initial antibodies to β-galactosidase. Each animal was injected i.m. with 100 micrograms of plasmid DNA per injection. Four separate injections were given at one week intervals.

Prior to the third injection, the animals were bled to monitor the early antibody response to β-gal. One week after the final injection of plasmid DNA, the animals were bled to monitor the late antibody response to beta-galactosidase. To test the sensitivity of the assay, known amounts of anti-β-gal antibodies isolated from an anti-β-gal antiserum were tested in parallel.

Serum dilutions of 1:40, 1:200, or 1:1000 were tested in an ELISA for anti-β-gal antibodies. For this, polystyrene microtiter ELISA plates were coated with β-gal at 10 microgram/ml in phosphate buffered saline. The plates were washed thrice with blocking buffer containing 1% bovine serum albumin (BSA), 0.2% Tween 20 in borate buffered saline (BBS) (0.1M borate, 0.2M NaCl, pH 8.2). 50 microliters of diluted serum were added to separate wells. After at least 1 hour at room temperature, the plates were washed thrice with blocking buffer and then allowed to react with alkaline phosphatase-conjugated goat anti-mouse IgG antibody. One hour later, the plates again were washed four times

with blocking buffer and incubated with 25 ml of TMB peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD). The absorbance at 405 nm of each well was measured using a microplate reader (Molecular devices, Menlo Park, CA). The higher the O.D. reading, the greater the amount of specific antibody in the sample.

The data for each of two experiments are provided in Tables IV and V which follow on separate sheets. The results are summarized in Tables VI and VII collating the data from the two experiments is provided as well. On the summary page n stands for the number of animals in each of the four groups. S.D. stands for standard deviation and Avg. is the average O.D. reading for all the animals in a particular group.

The results of Group 4 demonstrate that the use of a gene therapy vector encoding an accessory molecule ligand (CD40L) enhances the immunization against β-gal encoded by a genetic or gene therapy vector. The average O.D. reading of the 1:40 dilution of the sera from animals of this group is significantly higher than that of groups 1, 2, and 3 ($P < 0.05$, Bonferroni t tests, see Table VII).

Data from an additional experiment further reinforce the finding that the gene therapy vector encoding an accessory molecule ligand enhances immunization against β-gal (Figure 16). Here, pCD40L and placZ were co-injected into skeletal muscle, to test for enhancement of the immune response to placZ, a pcDNA3-based vector encoding *E. coli* β-galactosidase. The relative anti-β-gal Ab activities were determined via ELISA. As expected, mice injected with either the non-modified pcDNA3 vector or pCD40L alone did not produce detectable antibodies to b-gal (Figure 16A). Mice were injected with either 100 μg pcDNA3 (checkered bar), 50 μg pcDNA3 + 50 μg pCD40L (lined bar), 50 μg pcDNA3 + 50 μg placZ (striped bar), or 50 μg pCD40L + 50 μg placZ (solid bar). On the other hand, mice that

received placZ and pcDNA3 developed detectable anti-b-gal antibodies one week after the fourth and final injection, at d28. Mice that received placZ and pCD40L developed higher titers of anti-β-gal antibodies than mice injected with placZ and pcDNA3. Figure 16B, ELISA analyses of serial dilutions of sera collected at d28, shows that mice co-injected with placZ and pCD40L had an eight-fold higher mean titer of anti-β-gal antibodies at d28 than mice treated with placZ + pcDNA3.

i. Immunoglobulin Subclass Production Stimulated by Accessory Molecule Vector Co-Injection

Despite enhancing the titer of the anti-β-gal antibody response, the subclass of anti-β-gal IgG induced by injection of placZ was not altered by the co-injection of pCD40L. IgG_{2a} anti-β-gal antibodies predominated over IgG_1 subclass antibodies in the sera of mice injected with either placZ and pcDNA3 or placZ and pCD40L (Figure 17). Also depicted are the ELISA O.D. measurements of anti-β-gal IgG_1 and anti-β-gal IgG_{2a} present in the pre-immune sera (striped bar) or post-immune sera (solid bar), collected at d28) of each group of mice, injected as indicated on the abscissa. In contrast, BALB/c mice injected with β-gal protein developed predominantly IgG_1 anti-β-gal antibodies, and no detectable IgG_{2a} anti-β-gal antibodies.

ii. Augmentation of Vaccination by Accessory Molecule Vector Requires Co-Injection with placZ at the Same Site

The adjuvant effect of the pCD40L plasmid on the anti-β-gal antibody response was noted only when it was injected into the same site as placZ (Figure 18). Groups of BALB/c mice (n=4) received intramuscular injections of placZ and pCD40L together at the same site, or as simultaneous separate injections at distal

sites (right and left hind leg quadriceps). A control group received intramuscular injections of placZ and pcDNA3 at the same site. Animals were bled at d28 and the sera tested for anti-β-gal Ab at different dilutions, as indicated on the abscissa. The graph illustrates a representative experiment depicting the mean O.D. at 405 nm of replicate wells of each of the serum samples for each group, at a 1:40, 1:200, or 1:1000 dilution. Animals injected simultaneously with placZ and pCD40L, but at different sites, did not develop detectable anti-β-gal antibodies until d28. Moreover, the anti-β-gal antibody titers of the sera from such animals at d28 were similar to that of mice that received placZ and pcDNA3, and significantly less than that of animals that received placZ and pCD40L together at the same site.

iii. <u>Augmentation of Vaccination When Accessory Molecule Vector and placZ are Co-Injected into Dermis</u>

The pCD40L plasmid also enhanced the anti-b-gal antibody response to placZ when injected into the dermis. In the experiment shown in Figure 19, mice received intradermal injections, near the base of the tail, with either 50 μg pcDNA3 (checkered bar), 25 μg pcDNA3 + 25 μg pCD40L (lined bar), 25 μg pcDNA3 + 25 μg placZ (striped bar), or 25 μg pCD40L + 25 μg placZ (solid bar). Injections, bleeds and ELISA analyses were performed as in Figure 16A. The checkered bar and lined bar groups each consisted of 8 mice while the striped bar and solid bar groups each consisted of 12 mice. The height of each bar represents the mean O.D. of sera at a 1:40 dilution of each group ± S.E. A statistical analysis of the data indicated that the striped bar and solid bar groups are independent ($P < .05$). As observed with intramuscular injection, mice co-injected with placZ and pCD40L developed detectable serum anti-β-gal

antibodies one week following the second injection (d14), and two weeks earlier than mice injected with placZ and pcDNA3. Moreover, these animals also had an eight-fold higher mean titer of anti-β-gal antibodies than mice of the placZ-injected group at d28. Mice injected with either the non-modified pcDNA3 vector or pCD40L alone did not produce detectable antibodies to β-gal.

b. Augmentation of the CTL Response in Mice Co-Injected with an Accessory Molecule Gene Therapy Vector and placZ

The ability of pCD40L to enhance induction, by placZ, of CTL specific for syngeneic b-gal-expressing target cells was tested. BALB/c mice co-injected with pCD40L and placZ into skeletal muscle (Figure 20A) or dermis (Figure 20B) generated greater numbers of CTL specific for P13.2, a placZ transfected P815 cell line, than mice co-injected with placZ and pcDNA3. At a 5:1 effector:target ratio, the splenocyte effector cells from mice that received intramuscular injections of placZ and pCD40L achieved greater than 20% specific lysis of P13.2. In contrast, when splenocytes of mice that received the control injection with placZ and pcDNA3 were used, a 9-fold greater ratio of effector to target cells was required to achieve this level of specific lysis. Similarly, the splenocyte effector cells from mice that received intradermal injections of placZ and pCD40L killed more than 50% of the P13.2 cells at effector:target ratios of 4:1. To achieve comparable levels of specific lysis required eight-fold higher effector:target ratios using splenocytes from mice that received intradermal injections of placZ and pcDNA3. Nevertheless, the splenocytes of mice co-injected with pCD40L and placZ did not have greater non-specific CTL activity for P815 cells than that of mice that received placZ along with pcDNA3 (Figure 20). As expected, the

splenocytes from mice that received injections of pcDNA3 alone, or pcDNA3 and pCD40L, did not mediate specific lysis of P13.2 or P815 cells.

Table IV

Experiment #1 Injections of plasmid DNA i.m.: 4/3/96; 4/10/96; 4/17/96; 4/24/96

ELISA for anti-beta galactosidase antibodies:

Group	Animal	Dilution of Pre-Bleed (4/3) 1/140	1/200	1/1000	Dilution of Bleed (4/17) 1/140	1/200	1/1000	Dilution of Bleed (5/1) 1/40	1/200	1/1000
pcDNA3 (p-control, 100 mcg)	1	0.09	0.11	0.09	0.06	0.06	0.06	0.11	0.17	0.11
(Control vector)	2	0.11	0.09	0.09	0.07	0.07	0.07	0.10	0.09	0.08
	3	0.12	0.11	0.10	0.09	0.09	0.10	0.12	0.08	0.08
	4	0.11	0.10	0.10	0.08	0.11	0.07	0.11	0.07	0.08
	Avg.	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
	S.D.	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.04	0.01
p-lacZ (50 mcg)	5	0.13	0.10	0.10	0.07	0.11	0.06	0.15	0.10	0.08
+	6	0.10	0.11	0.10	0.07	0.06	0.06	0.22	0.15	0.14
p-Control (50 mcg)	7	0.19	0.10	0.18	0.07	0.07	0.06	0.78	0.29	0.12
	8	0.10	0.09	0.10	0.08	0.07	0.07	3.04	1.84	0.77
	Avg.	0.13	0.10	0.12	0.07	0.08	0.06	1.05	0.60	0.28
	S.D.	0.04	0.01	0.04	0.01	0.02	0.00	1.36	0.84	0.33
p-lacZ (50 mcg)	27	0.06	0.06	0.06	0.13	0.11	0.08	2.30	1.68	0.72
+	18	0.06	0.06	0.06	0.27	0.13	0.10	2.35	0.09	0.28
pRcCMV-mCD40L (p-mCD40L,	19	0.06	0.06	0.06	0.23	0.19	0.11	2.06	1.09	0.39
	20	0.06	0.06	0.06	0.23	0.19	0.11	2.06	1.09	0.39
	Avg.	0.06	0.06	0.06	0.74	0.47	0.21	2.25	1.00	0.47
	S.D.	0.00	0.00	0.00	1.06	0.66	0.24	0.13	0.67	0.19

Table V

Experiment #2 Injections of plasmid DNA i.m.: 6/5/96; 6/12/96; 6/19/96; 6/26/96

Dilutions of sera for anti-beta galactosidase antibodies:

		Dilution of Pre-Bleed (6/5)			Dilution of Bleed (7/19)			Dilution of Bleed (8/3)		
Group	Animal	1/140	1/200	1/1000	1/140	1/200	1/1000	1/40	1/200	1/1000
p-Control (50 mcg)	9	0.02	0.02	0.06	0.04	0.01	0.01	0.04	0.03	0.05
+	10	0.06	0.02	0.10	0.02	0.02	0.00	0.08	0.09	0.05
p-mCD40L (50 mcg)	11	0.02	0.02	0.07	0.03	0.01	0.00	0.02	0.02	0.05
	12	0.06	0.03	0.05	0.18	0.04	0.01	0.11	0.04	0.05
	Avg.	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
	S.D.	0.02	0.01	0.02	0.07	0.01	0.01	0.04	0.03	0.00
p-lacZ (50 mcg)	5	0.02	0.03	0.02	0.06	0.04	0.04	0.39	0.11	0.03
+	6	0.03	0.02	0.03	0.14	0.03	0.04	2.85	1.58	0.41
p-Control (50 mcg)	7	0.56	0.13	0.06	0.29	0.06	0.02	0.22	0.07	0.03
	8	0.01	0.02	0.05	0.06	0.02	0.02	0.11	0.04	0.05
	Avg.	0.15	0.05	0.04	0.13	0.04	0.03	0.89	0.45	0.13
	S.D.	0.27	0.05	0.02	0.11	0.02	0.01	1.31	0.75	0.19
p-lacZ (50 mcg)	13	0.23	0.06	0.05	0.28	0.07	0.02	2.37	0.73	0.18
+	14	0.02	0.02	0.03	0.04	0.02	0.01	3.05	2.23	0.59
p-mCD40L (50 mcg)	15	0.02	0.02	0.02	0.89	0.21	0.05	2.46	0.96	0.21
	16	0.05	0.04	0.02	0.11	0.04	0.04	2.75	1.39	0.34
	Avg.	0.08	0.04	0.03	0.33	0.08	0.03	2.66	1.33	0.33
	S.D.	0.10	0.02	0.02	0.39	0.09	0.02	0.31	0.67	0.19

Table VI

Summary

		Pre-Immune @ beta-gal			Early @ beta-gal			Late @ beta-gal		
		1/140	1/200	1/1000	1/140	1/200	1/1000	1/40	1/200	1/1000
1) p-Control (n = 4)	**Avg.**	**0.11**	**0.11**	**0.11**	**0.11**	**0.11**	**0.11**	**0.11**	**0.11**	**0.11**
	S.D.	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.04	0.01
2) p-mCD40L + p-Control (n = 4)	**Avg.**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**
	S.D.	0.02	0.01	0.02	0.07	0.01	0.01	0.04	0.03	0.00
3) p-lacZ + p-Control (n = 8)	**Avg.**	**0.11**	**0.04**	**0.04**	**0.11**	**0.03**	**0.03**	**0.61**	**0.31**	**0.09**
	S.D.	0.22	0.04	0.01	0.09	0.02	0.02	1.11	0.62	0.16
4) p-lacZ + p-mCD40L (n = 8)	**Avg.**	**0.11**	**0.04**	**0.03**	**0.25**	**0.06**	**0.03**	**2.06**	**1.04**	**0.26**
	S.D.	0.10	0.02	0.01	0.32	0.07	0.02	0.97	0.69	0.18

Anti-beta-galactosidase standard:	67 ng	22ng	7.4ng	2.5ng	.82ng	.27ng
O.D.	3.01	2.98	2.05	1.10	0.52	0.26
	3.14	3.14	2.25	1.20	0.56	0.26

Table VII

BONFERRONI t-TESTS

Comparison	Difference of means			t	P<.05
4 vs 2:	2.06 -	0.04 =	2.02	3.782	Yes
4 vs 1:	2.06 -	0.11 =	1.95	3.651	Yes
4 vs 3:	2.06 -	0.61 =	1.45	3.325	Yes
3 vs 2:	0.61 -	0.04 =	0.57	1.067	No
3 vs 1:	0.61 -	0.11 =	0.50	Do not test	
1 vs 2:	0.11 -	0.04 =	0.07	Do not test	

Degrees of freedom: 20

ONE WAY ANALYSIS OF VARIANCE

Group	N	Mean	Std Dev	SEM
1	4	0.11	0.01	0.00
2	4	0.04	0.04	0.02
3	8	0.61	1.11	0.39
4	8	2.06	0.97	0.34
5	4	1.51	0.77	0.38
6	4	1.14	0.53	0.26
7	4	0.83	0.43	0.22

ONE WAY ANALYSIS OF VARIANCE

Source of Variation	SS	DF	Variance Est (MS)
Between Groups	18.29	6	3.05
Within Groups	18.39	29	0.63
Total	36.69	35	

$$F = \frac{s2\_bet}{s2\_wit} = \frac{MSbet}{Mswit} = \frac{3.05}{0.63} = 4.81 \quad P = 0.002$$

4. <u>Treatment of Neoplasia Using a Gene Therapy Vector Containing an Accessory Molecule Gene or Chimeric Accessory Molecule Gene</u>

a. <u>Treatment of Neoplasia in Mice</u>

The treatment of a neoplasia in a mouse model system has been demonstrated using the genes encoding accessory molecule ligands of the present invention. Gene therapy vectors containing an accessory molecule ligand gene (murine CD40 ligand) were prepared as has been previously described in the above examples. These gene therapy vectors were used to introduce that accessory molecule ligand gene into neoplastic cells, Line1 cells, from a tumor which originated in BALB/c mice. The accessory molecules were introduced into the neoplastic cells according to the above examples. The expression of the accessory molecule ligand on the surface of these neoplastic cells was confirmed using flow cytometry as has been described in the above examples.

The effectiveness of the accessory molecule ligand genes for treating neoplasia was shown as follows. Female BALB/c mice (6-8 weeks old) were injected i.p. with 1.0 X 10^5 irradiated Line1 neoplastic cells. The neoplastic Line1 cells are derived from a spontaneous lung adenocarcinoma in a BALB/c mouse. This neoplastic cell has been described by Blieden et al., <u>Int. J. Cancer Supp.</u>, 6:82 (1991). Other female BALB/c mice were injected i.p. with 1.0 X 10^5 irradiated Line1 tumor cells that had previously been transduced with the gene therapy vector encoding the accessory molecule ligand gene (murine CD40) as described above.

Each group of mice was allowed to generate an immune response for 10 days. After 10 days each mouse was challenged with 1.0 X 10^4 live, non-irradiated Line1 neoplastic cells. These mice were then monitored for the formation of tumors and then sacrificed when the tumors grew to 2.0 cm because of morbidity. The results

of this monitoring are shown in Figure 7. As can be seen by Figure 7, the mice immunized with the neoplastic cell expressing the accessory molecule ligands of the present invention on the cell surface remained free of tumor throughout the experiment. Mice immunized with the neoplastic cells not having the accessory molecule ligand genes of the present invention succumbed to tumor 50 days after challenge with the neoplastic cells.

Figure 21 demonstrates downmodulation of human CD40L, but not murine CD40L, in lung tumor cell lines that express CD40. Human cell lines HeLa (CD40-negative cervical carcinoma, Figure 21A), A427 (CD40-negative lung carcinoma, Figure 21B), NCI 460 (weakly CD40-positive lung large cell carcinoma, Figure 21C), and SK-Mes-1 (strongly CD40-positive lung squamous cell tumor, Figure 21D) were infected with adenovirus encoding lac-Z (Ad-LacZ), murine CD40L (Ad-mCD40L), and human CD40L (Ad-hCD40L) at an MOI of 0 (Blank), 1, and 10. 48 hours after infection, murine CD40L and human CD40L surface expression was determined. The percentage of cells that express ligand are plotted on the Y-axis. Human and mouse CD40L are expressed at equal levels in CD40-negative cell lines. However, only murine CD40L expression is stable on cell lines that express CD40. In contrast to mCD40L, human CD40L is downmodulated on CD40-positive tumors.

The data graphed in Figure 22A show that CD40 binding induces expression of tumor surface markers. Treating CD40-expressing lung cancer cell lines with αCD40 mAb resulted in enhanced expression of the tumor cell surface markers CD95 (Fas), CD54 (ICAM-1) and class I major histocompatability antigens (MHC I). NCI 460, a weakly CD40-positive lung large cell carcinoma, was incubated with a CD40-specific monoclonal antibody (thick line), or MOPC21, an isotype control mAb (thin line), on CD32-expressing mouse fibroblasts for 48 hours. Following the 48 hr incubation, the lung tumor

cells were analyzed for CD95, CD54, and MHC-I expression by FACS.

Figure 22B again shows downmodulation of human CD40L by CD40-positive tumor cells. HeLa (CD40-negative), CLL (CD40-positive), and SK-MES-1 (CD40-positive) tumor cells were cocultured for 24 hours with CD3-activated normal donor T cells at a tumor cell:T cell ration of 2.5:1. Following coculture, CD2-expressing T cells were analyzed for CD40L surface expression by FACS. Thin lines represent T cells stained with FITC-labeled isotype control antibody (MOPC21) and thick lines represent activated T cells stained with FITC-labeled αCD40L antibody (αCD154 antibody). The CD40-positive tumor cell lines, SK-MES-1, and CLL, do not express CD40 ligand on their surfaces.

5. <u>Expression of the Human and Mouse Accessory Molecule Ligand, Fas Ligand, in Human Blood Lymphocytes</u>

a. <u>Construction of a Genetic Construct and Gene Therapy Vector Containing the Human and Mouse Fas Ligand Gene</u>

Either the human accessory molecule ligand gene (human Fas ligand) or the murine accessory molecule ligand gene (murine Fas ligand) was constructed utilizing the respective human and murine genes. An altered accessory cell molecule, in which a putative MMP-cleavage site was removed, was made and designated ΔFasL-pcDNA3. The nucleotide sequence of ΔFasL-pcDNA3 is listed as SEQ ID NO: 40. Human Fas ligand nucleotides 325 to 342, encoding six amino acids, are missing from ΔFasL. The design of ΔFasL was based on reasoning that Domain III contains sites most accessible to MMPs, and could thus be the target on the molecule for cleavage from the surface of the cell. Sequences of the human Fas ligand gene have been determined and are

listed as SEQ ID NOS: 13 and 30 (Genbank accession U11821). Sequences of mouse Fas ligand genes have been determined and are listed as SEQ ID NOS: 14 (C57BL/6, Genbank accession U10984) and 31 (Balb/c, Genbank accession U58995). The sequence of the rat Fas ligand gene has been determined and is listed as SEQ ID NO: 25 (Genbank accession U03470). Chimeric constructs are made, as described in Example 2 for CD40 ligand chimeric constructs, in which Domain III of human Fas ligand is replaced with Domains of other proteins, particularly proteins of the TNF family. Chimeric constructs include, but are not limited to, human Fas ligand with Domain III replaced by Domain III of murine Fas ligand (chimeric sequence listed as SEQ ID NO: 37, sequence line-up shown in Figure 37), or replaced by Domain III of human CD70 (chimeric sequence listed as SEQ ID NO: 38, sequence line-up shown in Figure 38), or replaced with Domain I of human CD70 (chimeric sequence listed as SEQ ID NO: 39, sequence line-up shown in Figure 39). Chimeric constructs in which multiple domains, for example, two copies of human CD70 Domain III, are inserted into human Fas ligand in place of Domain III, are also made using methods described in Example I. Chimeric constructs in which synthetic sequences are used to replace Domain III of human Fas ligand are also made.

i. <u>Human Fas Ligand Cloning</u>

The cDNA encoding human Fas-ligand was subcloned in the eukaryotic expression vector pcDNA3. Normal donor blood lymphocytes were activated for 4 hours with 1 ng/ml PMA plus 0.5 uM ionomycin. Total RNA was isolated with the Qiagen Rneasy kit. cDNA was then synthesized from poly-A RNA with oligo-dT primers using the Gibco-BRL Superscript cDNA synthesis kit. The gene encoding human Fas-ligand was then PCR amplified with the Fas-ligand-specific primers (sense primer, SEQ ID NO: 32,

antisense primer, SEQ ID NO: 33). The Fas-ligand PCR product was then subcloned into pcDNA3 using standard molecular biology techniques. RT-PCR products, subcloned into pcDNA3, are designated hFasL-pcDNA3.

ii. Murine Fas Ligand Cloning

The murine Fas-ligand geneS from Balb/c and C57/BL6 strains of mice were also amplified following activation of mouse splenocytes with PMA plus ionomycin as described above, and amplified from poly-A synthesized cDNA as described above (sense primer, SEQ ID NO: 34, antisense primer, SEQ ID NO: 35). These genes were subcloned in the pTARGET expression vector (Promega, Madison, WI). RT-PCR products, subcloned into pcDNA3, are designated mFasL-pcDNA3.

iii. Adenovirus Vector Construction

For construction of adenovirus vectors encoding human Fas-ligand, murine Fas-ligand or ΔFas-ligand, the cloned cDNA insert is subcloned into the plasmid pRc/RSV (Invitrogen, San Diego, CA) at the HindIII-Xbal site. A BglII-Xhol fragment with the RSV promoter-enhancer and the bovine growth hormone poly-A signal sequence was subcloned into the BamHI-Xhol site of plasmid MCS(SK) pXCX2. The plasmid MCS(SK)pXCX2 is a modification of the plasmid pXCX2, in which the pBluescript polylinker sequence was cloned into the E1 region. The resulting plasmid then is co-transfected along with pJM17 into 293 cells using the calcium phosphate method. Isolated plaques of adenovirus vectors are picked and expanded by infecting 293 cells. High titer adenovirus preparations are obtained, as described above which uses a cesium chloride gradient for concentrating virus particles via a step gradient, with the densities of 1.45g/cm^3 and 1.20g/cm^3, in which samples are centrifuged for 2 hours in an SW41 rotor (Beckman, Brea, CA) at 25,000 rpm at 4° C. The virus band is desalted using a Sephadex G-25 DNA

grade column (Pharmacia, Piscataway, NJ), and the isolated virus is stored at -70° C in phosphate-buffered saline with 10% glycerol. The titer of the virus is determined by infecting permissive 293 cells at various dilutions and counting the number of plaques. Titers typically range from 10^{10} to 10^{12} plaque forming units/ml. The adenovirus constructs are designated Ad-hFasL, Ad-mFasL and Ad-ΔFasL.

b. Introduction of the Murine and Human Fas Ligand Genes into Human Cells

The constructs hFasL-pcDNA3, mFasL-pcDNA3 and ΔFasL-pcDNA3 are transfected into 293 via electroporation. The transfected cells are selected in medium containing G418. Fas-ligand transfectants are screened for expression of the transgene using anti-Fas-ligand antibody and flow cytometry. The methods used are similar to those described for transfection of CD40L into CLL cells.

For FasL-adenovirus infection, 10^6 freshly thawed and washed CLL cells or HeLa cells are suspended in 0.5 to 1 mL of culture medium for culture at 37°C in a 5% CO_2-in-air incubator. Adenovirus are added to the cells at varying multiplicity of infection (MOI), and the infected cells are cultured for 48 hours, unless otherwise stated, before being analyzed for transgene expression.

c. Expression of the Fas Ligand Genes in Human Cells

Mice with the lymphoproliferative or generalized lymphoproliferative disorder are unable to delete activated self-reactive cells outside of the thymus. This is related to the fact that, in these mice, interactions between the Fas receptor and an accessory molecule ligand, Fas ligand, are defective. These animals develop numerous disorders including lymphadenopathy, splenomegaly, nephritis, and systemic

autoimmune pathology which resembles that seen in patients with systemic lupus erythematosus or rheumatoid arthritis (RA). It is conceivable that the normal interactions between the Fas receptor and the accessory molecule ligand that are responsible for clearance of activated lymphocytes from joints may be impaired in RA patients.

RA synovial lymphocytes express the Fas receptor at a higher proportion than that of matched RA blood lymphocytes to matched normal donor blood lymphocytes. On the other hand, RA synovial lymphocytes express little or no accessory molecule ligand. Since the RA synovial lymphocytes are sensitive to Fas-induced apoptosis, it is feasible that local expression of Fas ligand in the RA joint could serve to eliminate the synovial mononuclear cells that potentially mediate RA autoimmune pathology.

Figure 23 shows that Fas-ligand expression in lymphocytes is inhibited by exposure to RA synovial fluid. Normal donor blood T cells were activated for 5 hours with 1 ng/ml PMA plus 0.5 μM ionomycin. Cells were incubated in the presence of rheumatoid arthritis blood plasma (circles), RA synovial fluid (diamonds), or neither (squares). In addition, cells were incubated with increasing concentrations of the MMP inhibitor BB94. Following activation, cells were analyzed for Fas-ligand surface expression by FACS. The percentage of cells expressing Fas ligand are plotted in Figure 23. This experiment demonstrates that there is a factor(s) present in RA synovial fluid and serum that prevents surface expression of Fas-ligand.

d. <u>Function of Human, Murine and Chimeric Accessory Molecule Ligand, Fas Ligand</u>

To determine the capacity of the ΔFasL constructs, the above-mentioned transfected cells are mixed with the Fas-ligand sensitive human T cell line, JURKAT. Following 4 hours coculture, the nonadherant JURKAT cells

are collected and evaluated for apoptosis. The fluorescent compound 3,3' dihexyloxacarbocyanine iodide ($DiOC_6$) is used to evaluate for apoptosis using a modification of a previously described protocol. For this, the cells are washed once at room temperature in phosphate buffered saline (PBS, pH 7.2). Cells are placed into separate wells of a 96 well U-bottom plastic microtiter plate at 10^5 - 5×10^5 cells/well in 50 ml total volume. If indicated, saturating amounts of PE-conjugated antibodies are added followed by addition of $DiOC_6$ and propidium iodide (PI). $DiOC_6$ and PI are used at 40 nM and 10 ng/ml final concentrations, respectively. The cells are then incubated 15 minutes in a 37°C, 5% CO_2 tissue culture incubator. The stained cells are then washed twice in ice cold PBS and ultimately suspended in 200 ml SM and analyzed by FACS. Dead cells and debris with characteristic forward and light scatter profiles and PI staining are excluded from analysis.

The ability of cells expressing ΔFasL-pcDNA3 to direct Fas-mediated apoptosis of cells expressing CD95 is compared with that of cells expressing FasL-pcDNA3. Relative stability of the protein products encoded by ΔFasL-pcDNA3 or FasL-pcDNA3 pre- and post- culture with RA synovial fluid, and with or without the metallo-proteinase inhibitors, are assessed via flow cytometry of cells expressing either ligand.

6. Treatment of Arthritis with Gene Therapy Vectors Encoding an Accessory Molecule Ligand, Fas Ligand

The heterologous Fas-ligand constructs, made as described above, that show the highest stability of expression in combination with the greatest ability to mediate Fas-induced apoptosis, are used in gene therapy for RA. Potential therapeutic constructs are tested in well-characterized mouse models of arthritis to assess efficacy and function *in vivo*.

a. Gene Therapy Treatment of Arthritis in Mice

i. Mouse Models for Arthritis

One mouse arthritis model is collagen-induced arthritis. It is known that injecting DBA/1 mice with type II collagen in complete Freund's adjuvant (CFA) induces an arthritis with synovitis and erosions that histologically resemble RA. For our studies, male DBA/I mice are immunized with bovine type II collagen in complete Freund's adjuvant on day 0 and boosted itraperitoneally (i.p.) on day 21. On day 28, animals are given an additional i.p. injection with lipopolysaccharide (LPS) and/or the same type collagen, or an injection of acetic acid alone. Swelling and/or redness of a fore or hind paw in animals immunized with collagen typically is detected the third or fourth week following the second injection. The vertebrae are only rarely affected, and then only weeks after the initial peripheral joint swelling. Affected joints display initial histologic changes of synovial edema, followed by synovial hyperplasia.

Another animal model, recently described by Kouskoff, V. et al., in Cell 87:811-822 (1997) was generated fortuitously, by crossing a T cell receptor (TCR) transgenic mouse line with the non-obese-diabetic (NOD) strain to produce the KRN x NOD mouse model of RA. The offspring of such a mating universally develop a joint disease that is highly similar to that of patients with RA. Moreover, the disease in these animals has an early and reproducible time of onset and a highly reproducible course. The arthritis apparently is induced by chance recognition of an NOD-derived major histocompatibility complex (MHC) class II molecule by the transgenic TCR, leading to breakdown in the general mechanisms of self-tolerance and systemic self-reactivity.

ii. Relief of Arthritis Symptoms in Mice Treated with a Gene Therapy Vector Encoding an Accessory Molecule Ligand

We have adapted and modified a protocol originally described by Sawchuk and colleagues for micro-injecting adenovirus vectors into mouse joints. Using this procedure we can reproducibly inject a 5 μl volume into the articular space of the mouse knee. In this procedure, the mice are anesthetized with metofane. A small incision of approximately 2-3 mm is made with a #11 scalpel blade in the skin over the lateral aspect of the knee to visualize the patello-tibial ligament. We can inject up to 5 μl of fluid using a micro-100 μl-Hamilton syringe and a 30-gauge needle. After the injection, the knee incision is closed with Nexabond (Veterinary Products Laboratory). Our adenovirus titers typically exceed 10^{10} plaque forming units (pfu) per ml, making it possible to deliver at least 5 x 10^8 pfu of virus in 5 ml into the knee joints, as outlined above. Control animals are injected with control Ad-lacZ vector, a replication-defective adenovirus vector lacking a transgene, or with the buffer used to suspend the virus (10 mM Tris, 1 mM $MgCl_2$, 10% glycerol).

In another method, splenocytes will be harvested from mice that are syngeneic to the host animal intended for adoptive transfer of transduced cells. Cell proliferation will be induced with exogenous IL-12 (100 units/ml) for 48 h. Cells are counted and then re-plated at densities of 5 x 10^5 or 1 x 10^6 cells per ml in a 12-well dish with 1 ml complete culture medium per well. Virus and ConA are added together at the time of re-plating in the presence of polybrene (8 μg/ml). The medium is changed 24 hours after infection with complete medium containing 100 units of recombinant IL-2 per ml. Aliquots of the transduced cells are examined, for Fas-ligand expression, at 48 hours after infection via flow cytometry.

Animals will receive standardized numbers of cytokine-producing cells or control mock-transfected cells intraperitoneally. Concentrated cell suspensions are injected directly into the mouse synovium, as described in section 4A above. In parallel, aliquots of the transferred cell populations are maintained in tissue culture supplemented with exogenous IL-2.

Mice are monitored in a blinded fashion for signs of arthritis. The date of disease onset is recorded and clinical severity of each joint or group of joints (toes, tarsus, ankle, wrist, knee) are graded as follows: 0 (normal), 1 (erythema), 2 (swelling), 3 (deformity), 4 (necrosis). The scores are summed to yield the arthritic score. The severity of arthritis is expressed both as the mean score observed on a given day, and as the mean of the maximal arthritic score reached by each mouse during the clinical course of the disease. At the time of death, hind paws are dissected free and processed for histologic examination or for RT-PCR. The histologic severity of the arthritis is scored on a scale of 0-3 for synovial proliferation and inflammatory cell infiltration, where a score of 0 = normal and 3 = severe.

For mice receiving intra-synovial injection of control of test adenovirus vector, the level of arthritis observed between contralateral sites is compared. In addition, the overall joint score minus that of the injected joint for the entire animal is compared with that observed in the joint injected with the control or test adenovirus vector.

Local administration of Fas-ligand adenovirus expression vectors will result in clearance of activated cells, as assessed by measuring the relative levels of CD80 mRNA by quantitative RT-PCR. This treatment also will lead to an enhanced level. Also, whether such level of apoptosis identified in affected mouse synovial tissue is assessed by the TUNEL assay ("Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End

Labeling"). TUNEL is performed by immersing the sections in TdT buffer (30 mM Tris-HCl, pH 7.2, 140 nM sodium cacodylate, 1 mM cobalt chloride), and then adding TdT (GIBCO BRL, Grand Island, NY) and biotinylated dUTP (Boehringer Mannheim, Indianapolis, IN). The reaction is terminated by immersing the sections in TB buffer (300 mM sodium chloride, 30 mM sodium citrate). Subsequently, the samples are treated with peroxidase-labeled streptavidin and then visualized using the VECTASTAIN ABC kit (Vector Laboratories Inc., Burlingame, CA). For immunohistochemistry, the sections are blocked with 4% skim milk for 30 minutes at room temperature, then incubated with biotinylated mAbs specific for mouse CD3, B220, CD80, or CD95 (Fas). These antibodies are available from Pharmingen (San Diego, CA).

b. Treatment of Rheumatoid Arthritis Patients with a Gene Therapy Vector Encoding an Accessory Molecule Ligand, Fas Ligand

Candidate Fas-ligand constructs identified as having potential therapeutic benefit are used in human protocols to treat RA. Human protocols encompass either *in vivo* or *ex vivo* methods to deliver the Fas-ligand constructs. Furthermore, the Fas-ligand constructs are potentially delivered by either viral or non-viral methods. Outlines of therapeutic strategies are described below.

An *ex vivo* therapy is similar to a protocol described for intra-articular transplantation of autologous synoviocytes retrovirally transduced to synthesize interleukin-1 receptor antagonist (Evan, Christopher et. al., Clinical Trial to Assess the Safety, Feasibility, and Efficacy of Transferring a Potentially Anti-Arthritic Cytokine Gene to Human Joints with Rheumatoid arhtritis, Human Gene Therapy, Vol. 7, 1261-1280). In this procedure, after clinical diagnosis of RA, the synovium is harvested during total joint replacement. The synoviocytes re-isolated and expanded,

then transduced or transfected with heterologous Fas-ligand into synoviocytes (via retrovirus, adenovirus, naked DNA, etc.). The gene-modified synoviocytes are then reinjected into the patient, who is monitored and tested for amelioration of RA-associated symptoms, and for expression and function of the Fas-ligand in modified synoviocytes.

In another <u>ex vivo</u> protocol, an allogeneic immortalized cell line that stably expresses the heterologous Fas-ligand is administered to the RA patient. In this protocol, a stable immortalized cell line expressing Fas-ligand (introduced by transfection of the gene into the cell by nonviral methods, such as electroporation), or by viral transduction of the gene into the cell) is constructed. The modified cell line is injected into the patient, who is monitored and tested for amelioration of RA associated symptoms, and for expression and function of the hFas-ligand in modified synoviocytes.

An <u>in vivo</u> based therapy will is similar in concept to the amelioration of collagen-induced-arthritis using a murine Fas-ligand adenovirus gene therapy vector, described in Zhang, et al., <u>J. Clin. Invest.</u> 100:1951-1957 (1997). In our use of such an approach, delivery of the hFas-ligand construct or chimeric ΔfasL directly to the joints of RA patients is performed using either viral or non-viral methods. In this procedure, the Fas-ligand construct (e.g. hFas-ligand adenovirus) is directly injected into the synovium. Patients are monitored and tested for amelioration of RA-associated symptoms as well as biological testing for expression and function of the hFas-ligand in modified synoviocytes.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Kipps, Thomas J.
Sharma, Sanjai
Cantwell, Mark

(ii) TITLE OF INVENTION: NOVEL EXPRESSION VECTORS CONTAINING ACCESSORY MOLECULE LIGAND GENES AND THEIR USE FOR IMMUNOMODULATION AND TREATMENT OF MALIGNANCIES AND AUTOIMMUNE DISEASE

(iii) NUMBER OF SEQUENCES: 35

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(C) CITY: Los Angeles
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(E) COUNTRY: U.S.A.
(F) ZIP: 90071-2066

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette,
1.44 Mb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: IBM P.C. DOS 5.0
(D) SOFTWARE: FastSeq Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: To Be Assigned
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 60/132145
(B) FILING DATE: 12/9/96

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Guise, Jeffrey W.
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(C) REFERENCE/DOCKET NUMBER: 231/003

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 786 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 783 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 783 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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TGA                                                                  783
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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 786 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 783 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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TTGCTGAACT GTGAGGAGAT GAGAAGGCAA TTTGAAGACC TTGTCAAGGA TATAACGTTA   300
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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 786 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 786 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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GAAGATTTTG TATTCATGAA AACGATACAG AGATGCAACA CAGGAGAAAG ATCCTTATCC    240
TTACTGAACT GTGAGGAGAT TAAAAGCCAG TTTGAAGGCT TTGTGAAGGA TATAATGTTA    300
AACAAAGAGG AGACGAAGAA AGAAAACAGC TTTGAAATGC AAAAAGGTGA TCAGAATCCT    360
CAAATTGCGG CACATGTCAT AAGTGAGGCC AGCAGTAAAA CAACATCTGT GTTACAGTGG    420
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TTCGAGAGAA TCTTACTCAG AGCTGCAAAT ACCCACAGTT CCGCCAAACC TTGCGGGCAA    660
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CTCTGA                                                               786
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(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 864 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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AACCATGCGG GCAGCAATCC ATTCACTTAG GAGGAGTCTT TGAATTGCAA TCGGGTGCTT    720
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(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3634 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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GCGCTCCCCA AGAAGACAGG GGGGCCCCAG GGCTCCAGGC GGTGCTTGTT CCTCAGCCTC 900
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(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1997 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

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CCATGTTGGC CAGCTGGTCT CAAACTCCTG ACCTCAAGTA ATCCGCCCAC CTCAGACTCC 240
CAAAGTGCCA GGATTACAGG TGTGAGCCAC TGCACCAGGC CTGGAACAAT TTTAAAATAA 300

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CACAGCACCC TCAAACCTGC TGCTCACCTC ATTGGTAAAC ATCCACCTGA CCTCCCAGAC   1980
ATGTCCCCAC CAGCTCT                                                  1997
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(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10240 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

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CCCCTCCCAC ACAGGGAAAG CCCCAAACCA ACTGCTGGCC TCCTCAAGAA AGAAACCGAA    240
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TCTGTCTCTC ATGTCTTCTC ATCACTCCTT CTCACCTTCC CGTTTTTGTC CTGCAATGCC    420
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CTGGATGCAA CCTGGGACAT CTGGAATGTG GCTGCCAGGG AAGCTTGGGT TCCAATCGGA    9360
ATACTTCAGA ACATTCCTTG AGAAGATTTC ACCTCAATCT TGATGACTTT TTAGGCTTCC    9420
CTTTCTTCCA ATTTTCCAGA CTTCCCTGGG ATGGGGAGCC CAGCCCCAAA CCCCACAGGC    9480
CAGCTCCCTC TTATTTATAT TTGCACTTGG CATTATTATT TATTTATTTA TTTATTATTT    9540
ATTTACTAGT GAATGTATTT ATTCAGGAGG GCGAGGTGTC CTGGGAGACC CAGCATAAGG    9600
GCTGCCTTGG TTCAGATGTG TTTTCTGTGA AAACGGAGCT GAACTGTAGG TTGCTCCCAC    9660
CTGGCCTCCT AGCCTCTGTG CCTCCTTTTG CTTATGTTTT TAAAAACAAA TATTTATCTG    9720
ATCGAGTTGT CTAAATAATG CTGATTTGGT GACTAACTTG TCGCTACATC GCTGAACCTC    9780
TGCTCCCCAG GGGAGTTGTG TCTGTAACCG CCCTACTGGT CAGTGGCGAG AAATAAAAGC    9840
GTGCTTAGAA AAGAAATCTG GCCTCTTTCT GCGACTGAAT TCTGCATCTC CTTGGGGGGG    9900
TGAGGCTGCT CCCCAAAATT CTTTCTCCAC CGGGCTTAGG ATTCCCTGGG CTTCACTCCT    9960
GAGCTTGGAC TGCCTGGCTC AGGAGCCTCT GCAAGAAACA AAGCCCAGCC AAACAGGTCC   10020
CTCCCCTAAG AAAGGAACCT GAAGGTAATT ACCTCTCCCT CAGGGTGTGG GAATTTCCAA   10080
GTCTGGGAAT TCCTATCCAG CTGGGGAAGT CTGCAGTGCA GGTGAGACTT CCGGCTGAAA   10140
GAGCCAGGGA GCGGCCAGAT GCTCAGGTAC CTGAACCAGA GCCAAGGGAC TTCCAGACAG   10200
TGAGGCAACT GGGCTCCAAA TAACCTGATC CGGGGAATTC                          10240
```

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1644 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
CCTCAGCGAG GACAGCAAGG GACTAGCCAG GAGGGAGAAC AGAAACTCCA GAACATCCTG    60
GAAATAGCTC CCAGAAAAGC AAGCAGCCAA CCAGGCAGGT TCTGTCCCTT TCACTCACTG   120
GCCCAAGGCG CCACATCTCC CTCCAGAAAA GACACCATGA GCACAGAAAG CATGATCCGC   180
GACGTGGAAC TGGCAGAAGA GGCACTCCCC CAAAAGATGG GGGGCTTCCA GAACTCCAGG   240
CGGTGCCTAT GTCTCAGCCT CTTCTCATTC CTGCTTGTGG CAGGGGCCAC CACGCTCTTC   300
TGTCTACTGA ACTTCGGGGT GATCGGTCCC CAAAGGGATG AGAAGTTCCC AAATGGCCTC   360
CCTCTCATCA GTTCTATGGC CCAGACCCTC ACACTCAGAT CATCTTCTCA AAATTCGAGT   420
GACAAGCCTG TAGCCCACGT CGTAGCAAAC CACCAAGTGG AGGAGCAGCT GGAGTGGCTG   480
AGCCAGCGCG CCAACGCCCT CCTGGCCAAC GGCATGGATC TCAAAGACAA CCAACTAGTG   540
GTGCCAGCCG ATGGGTTGTA CCTTGTCTAC TCCCAGGTTC TCTTCAAGGG ACAAGGCTGC   600
CCCGACTACG TGCTCCTCAC CCACACCGTC AGCCGATTTG CTATCTCATA CCAGGAGAAA   660
GTCAACCTCC TCTCTGCCGT CAAGAGCCCC TGCCCCAAGG ACACCCCTGA GGGGGCTGAG   720
CTCAAACCCT GGTATGAGCC CATATACCTG GGAGGAGTCT TCCAGCTGGA GAAGGGGGAC   780
CAACTCAGCG CTGAGGTCAA TCTGCCCAAG TACTTAGACT TTGCGGAGTC CGGGCAGGTC   840
TACTTTGGAG TCATTGCTCT GTGAAGGGAA TGGGTGTTCA TCCATTCTCT ACCCAGCCCC   900
CACTCTGACC CCTTTACTCT GACCCCTTTA TTGTCTACTC CTCAGAGCCC CCAGTCTGTG   960
TCCTTCTAAC TTAGAAAGGG GATTATGGCT CAGAGTCCAA CTCTGTGCTC AGAGCTTTCA  1020
ACAACTACTC AGAAACACAA GATGCTGGGA CAGTGACCTG GACTGTGGGC CTCTCATGCA  1080
CCACCATCAA GGACTCAAAT GGGCTTTCCG AATTCACTGG AGCCTCGAAT GTCCATTCCT  1140
GAGTTCTGCA AAGGGAGAGT GGTCAGGTTG CCTCTGTCTC AGAATGAGGC TGGATAAGAT  1200
CTCAGGCCTT CCTACCTTCA GACCTTTCCA GACTCTTCCC TGAGGTGCAA TGCACAGCCT  1260
TCCTCACAGA GCCAGCCCCC CTCTATTTAT ATTTGCACTT ATTATTTATT ATTTATTTAT  1320
TATTTATTTA TTTGCTTATG AATGTATTTA TTTGGAAGGC CGGGGTGTCC TGGAGGACCC  1380
AGTGTGGGAA GCTGTCTTCA GACAGACATG TTTTCTGTGA AAACGGAGCT GAGCTGTCCC  1440
CACCTGGCCT CTCTACCTTG TTGCCTCCTC TTTTGCTTAT GTTTAAAACA AAATATTTAT  1500
CTAACCCAAT TGTCTTAATA ACGCTGATTT GGTGACCAGG CTGTCGCTAC ATCACTGAAC  1560
CTCTGCTCCC CACGGGAGCC GTGACTGTAA TTGCCCTACA GTCAATTGAG AGAAATAAAG  1620
ATCGCTTAAA ATAAAAAACC CCCC                                         1644
```

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1890 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
AAACAGAGAG AGATAGAGAA AGAGAAAGAC AGAGGTGTTT CCCTTAGCTA TGGAAACTCT    60
ATAAGAGAGA TCCAGCTTGC CTCCTCTTGA GCAGTCAGCA ACAGGGTCCC GTCCTTGACA   120
CCTCAGCCTC TACAGGACTG AGAAGAAGTA AAACCGTTTG CTGGGGCTGG CCTGACTCAC   180
CAGCTGCCAT GCAGCAGCCC TTCAATTACC CATATCCCCA GATCTACTGG GTGGACAGCA   240
GTGCCAGCTC TCCCTGGGCC CCTCCAGGCA CAGTTCTTCC CTGTCCAACC TCTGTGCCCA   300
GAAGGCCTGG TCAAAGGAGG CCACCACCAC CACCGCCACC GCCACCACTA CCACCTCCGC   360
CGCCGCCGCC ACCACTGCCT CCACTACCGC TGCCACCCCT GAAGAAGAGA GGGAACCACA   420
GCACAGGCCT GTGTCTCCTT GTGATGTTTT TCATGGTTCT GGTTGCCTTG GTAGGATTGG   480
GCCTGGGGAT GTTTCAGCTC TTCCACCTAC AGAAGGAGCT GGCAGAACTC CGAGAGTCTA   540
CCAGCCAGAT GCACACAGCA TCATCTTTGG AGAAGCAAAT AGGCCACCCC AGTCCACCCC   600
CTGAAAAAAA GGAGCTGAGG AAAGTGGCCC ATTTAACAGG CAAGTCCAAC TCAAGGTCCA   660
TGCCTCTGGA ATGGGAAGAC ACCTATGGAA TTGTCCTGCT TTCTGGAGTG AAGTATAAGA   720
AGGGTGGCCT TGTGATCAAT GAAACTGGGC TGTACTTTGT ATATTCCAAA GTATACTTCC   780
GGGGTCAATC TTGCAACAAC CTGCCCCTGA GCCACAAGGT CTACATGAGG AACTCTAAGT   840
```

```
ATCCCCAGGA TCTGGTGATG ATGGAGGGGA AGATGATGAG CTACTGCACT ACTGGGCAGA     900
TGTGGGCCCG CAGCAGCTAC CTGGGGGCAG TGTTCAATCT TACCAGTGCT GATCATTTAT     960
ATGTCAACGT ATCTGAGCTC TCTCTGGTCA ATTTTGAGGA ATCTCAGACG TTTTTCGGCT    1020
TATATAAGCT CTAAGAGAAG CACTTTGGGA TTCTTTCCAT TATGATTCTT TGTTACAGGC    1080
ACCGAGAATG TTGTATTCAG TGAGGGTCTT CTTACATGCA TTTGAGGTCA AGTAAGAAGA    1140
CATGAACCAA GTGGACCTTG AGACCACAGG GTTCAAAATG TCTGTAGCTC CTCAACTCAC    1200
CTAATGTTTA TGAGCCAGAC AAATGGAGGA ATATGACGGA AGAACATAGA ACTCTGGGCT    1260
GCCATGTGAA GAGGGAGAAG CATGAAAAAG CAGCTACCCA GGTGTTCTAC ACTCATCTTA    1320
GTGCCTGAGA GTATTTAGGC AGATTGAAAA GGACACCTTT TAACTCACCT CTCAAGGTGG    1380
GCCTTGCTAC CTCAAGGGGG ACTGTCTTTC AGATACATGG TTGTGACCTG AGGATTTAAG    1440
GGATGGAAAA GGAAGACTAG AGGCTTGCAT AATAAGCTAA AGAGGCTGAA AGAGGCCAAT    1500
GCCCCACTGG CAGCATCTTC ACTTCTAAAT GCATATCCTG AGCCATCGGT GAAACTAACA    1560
GATAAGCAAG AGAGATGTTT TGGGGACTCA TTTCATTCCT AACACAGCAT GTGTATTTCC    1620
AGTGCCAATT GTAGGGGTGT GTGTGTGTGT GTGTGTGTGT GTGTATGACT AAAGAGAGAA    1680
TGTAGATATT GTGAAGTACA TATTAGGAAA ATATGGGTTG CATTTGGTCA AGATTTTGAA    1740
TGCTTCCTGA CAATCAACTC TAATAGTGCT TAAAAATCAT TGATTGTCAG CTACTAATGA    1800
TGTTTTCCTA TAATATAATA AATATTTATG TAGATGTGCA TTTTTGTGAA ATGAAAACAT    1860
GTAATAAAAA GTATATGTTA GGATACAAAT                                     1890
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1541 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
GGGTGTCTCA CAGAGAAGCA AAGAGAAGAG AACAGGAGAA ATGGTGTTTC CCTTGACTGC      60
GGAAACTTTA TAAAGAAAAC TTAGCTTCTC TGGAGCAGTC AGCGTCAGAG TTCTGTCCTT     120
GACACCTGAG TCTCCTCCAC AAGGCTGTGA GAAGGAAACC CTTTCCTGGG GCTGGGTGCC     180
ATGCAGCAGC CCATGAATTA CCCATGTCCC CAGATCTTCT GGGTAGACAG CAGTGCCACT     240
TCATCTTGGG CTCCTCCAGG GTCAGTTTTT CCCTGTCCAT CTTGTGGGCC TAGAGGGCCG     300
GACCAAAGGA GACCGCCACC TCCACCACCA CCTGTGTCAC CACTACCACC GCCATCACAA     360
CCACTCCCAC TGCCGCCACT GACCCCTCTA AAGAAGAAGG ACCACAACAC AAATCTGTGG     420
CTACCGGTGG TATTTTTCAT GGTTCTGGTG GCTCTGGTTG GAATGGGATT AGGAATGTAT     480
CAGCTCTTCC ACCTGCAGAA GGAACTGGCA GAACTCCGTG AGTTCACCAA CCAAAGCCTT     540
AAAGTATCAT CTTTTGAAAA GCAAATAGCC AACCCCAGTA CACCCTCTGA AAAAAAAGAG     600
CCGAGGAGTG TGGCCCATTT AACAGGGAAC CCCCACTCAA GGTCCATCCC TCTGGAATGG     660
GAAGACACAT ATGGAACCGC TCTGATCTCT GGAGTGAAGT ATAAGAAAGG TGGCCTTGTG     720
ATCAACGAAA CTGGGTTGTA CTTCGTGTAT TCCAAAGTAT ACTTCCGGGG TCAGTCTTGC     780
AACAACCAGC CCCTAAACCA CAAGGTCTAT ATGAGGAACT CTAAGTATCC TGAGGATCTG     840
GTGCTAATGG AGGAGAAGAG GTTGAACTAC TGCACTACTG GCCAGATATG GGCCCACAGC     900
AGCTACCTGG GGGCAGTATT CAATCTTACC AGTGCTGACC ATTTATATGT CAACATATCT     960
CAACTCTCTC TGATCAATTT TGAGGAATCT AAGACCTTTT TCGGCTTGTA TAAGCTTTAA    1020
AAGAAAAAGC ATTTTAAAAT GATCTACTAT TCTTTATCAT GGGCACCAGG AATATTGTCT    1080
TGAATGAGAG TCTTCTTAAG ACCTATTGAG ATTAATTAAG ACTACATGAG CCACAAAGAC    1140
CTCATGACCG CAAGGTCCAA CAGGTCAGCT ATCCTTCATT TTCTCGAGGT CCATGGAGTG    1200
GTCCTTAATG CCTGCATCAT GAGCCAGATG GAAGGAGGTC TGTGACTGAG GGACATAAAG    1260
CTTTGGGCTG CTGTGTAGCA ATGCAGAGGC ACAGAGAAAG AACTGTCTGA TGTTAAATGG    1320
CCAAGAGAAT TTTAACCATT GAAGAAGACA CCTTTACACT CACTTCCAGG GTGGGTCTAC    1380
TTACTACCTC ACAGAGGCCG TTTTTGAGAC ATAGTTGTGG TATGAATATA CAAGGGTGAG    1440
AAAGGAGGCT CATTTGACTG ATAAGCTAGA GACTGAAAAA AAGACAGTGT CTCATTGGCA    1500
CCATCTTTAC TGTTACCTGA TGTTTTCTGA GCCGACCTTT G                        1541
```

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 888 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
GGCTGGTCCC CTGACAGGTT GAAGCAAGTA GACGCCCAGG AGCCCCGGGA GGGGGCTGCA     60
GTTTCCTTCC TTCCTTCTCG GCAGCGCTCC GCGCCCCCAT CGCCCCTCCT GCGCTAGCGG    120
AGGTGATCGC CGCGGCGATG CCGGAGGAGG GTTCGGGCTG CTCGGTGCGG CGCAGGCCCT    180
ATGGGTGCGT CCTGCGGGCT GCTTTGGTCC CATTGGTCGC GGGCTTGGTG ATCTGCCTCG    240
TGGTGTGCAT CCAGCGCTTC GCACAGGCTC AGCAGCAGCT GCCGCTCGAG TCACTTGGGT    300
GGGACGTAGC TGAGCTGCAG CTGAATCACA CAGGACCTCA GCAGGACCCC AGGCTATACT    360
GGCAGGGGGG CCCAGCACTG GGCCGCTCCT TCCTGCATGG ACCAGAGCTG GACAAGGGGC    420
AGCTACGTAT CCATCGTGAT GGCATCTACA TGGTACACAT CCAGGTGACG CTGGCCATCT    480
GCTCCTCCAC GACGGCCTCC AGGCACCACC CCACCACCCT GGCCGTGGGA ATCTGCTCTC    540
CCGCCTCCCG TAGCATCAGC CTGCTGCGTC TCAGCTTCCA CCAAGGTTGT ACCATTGCCT    600
CCCAGCGCCT GACGCCCCTG GCCCGAGGGG ACACACTCTG CACCAACCTC ACTGGGACAC    660
TTTTGCCTTC CCGAAACACT GATGAGACCT TCTTTGGAGT GCAGTGGGTG CGCCCCTGAC    720
CACTGCTGCT GATTAGGGTT TTTTAAATTT TATTTTATTT TATTTAAGTT CAAGAGAAAA    780
AGTGTACACA CAGGGGCCAC CCGGGGTTGG GGTGGGAGTG TGGTGGGGGG TAGTGGTGGC    840
AGGACAAGAG AAGGCATTGA GCTTTTTCTT TCATTTTCCT ATTAAAAA                 888
```

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1906 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
CCAAGTCACA TGATTCAGGA TTCAGGGGGA GAATCCTTCT TGGAACAGAG ATGGGCCCAG     60
AACTGAATCA GATGAAGAGA GATAAGGTGT GATGTGGGGA AGACTATATA AAGAATGGAC    120
CCAGGGCTGC AGCAAGCACT CAACGGAATG GCCCCTCCTG GAGACACAGC CATGCATGTG    180
CCGGCGGGCT CCGTGGCCAG CCACCTGGGG ACCACGAGCC GCAGCTATTT CTATTTGACC    240
ACAGCCACTC TGGCTCTGTG CCTTGTCTTC ACGGTGGCCA CTATTATGGT GTTGGTCGTT    300
CAGAGGACGG ACTCCATTCC CAACTCACCT GACAACGTCC CCCTCAAAGG AGGAAATTGC    360
TCAGAAGACC TCTTATGTAT CCTGAAAAGA GCTCCATTCA AGAAGTCATG GGCCTACCTC    420
CAAGTGGCAA AGCATCTAAA CAAAACCAAG TTGTCTTGGA ACAAAGATGG CATTCTCCAT    480
GGAGTCAGAT ATCAGGATGG GAATCTGGTG ATCCAATTCC CTGGTTTGTA CTTCATCATT    540
TGCCAACTGC AGTTTCTTGT ACAATGCCCA AATAATTCTG TCGATCTGAA GTTGGAGCTT    600
CTCATCAACA AGCATATCAA AAAACAGGCC CTGGTGACAG TGTGTGAGTC TGGAATGCAA    660
ACGAAACACG TATACCAGAA TCTCTCTCAA TTCTTGCTGG ATTACCTGCA GGTCAACACC    720
ACCATATCAG TCAATGTGGA TACATTCCAG TACATAGATA CAAGCACCTT TCCTCTTGAG    780
AATGTGTTGT CCATCTTCTT ATACAGTAAT TCAGACTGAA CAGTTTCTCT TGGCCTTCAG    840
GAAGAAAGCG CCTCTCTACC ATACAGTATT TCATCCCTCC AAACACTTGG GCAAAAAGAA    900
AACTTTAGAC CAAGACAAAC TACACAGGGT ATTAAATAGT ATACTTCTCC TTCTGTCTCT    960
TGGAAAGATA CAGCTCCAGG GTTAAAAAGA GAGTTTTTAG TGAAGTATCT TTCAGATAGC   1020
AGGCAGGGAA GCAATGTAGT GTGGTGGGCA GAGCCCCACA CAGAATCAGA AGGGATGAAT   1080
GGATGTCCCA GCCCAACCAC TAATTCACTG TATGGTCTTG ATCTATTTCT TCTGTTTTGA   1140
GAGCCTCCAG TTAAAATGGG GCTTCAGTAC CAGAGCAGCT AGCAACTCTG CCCTAATGGG   1200
AAATGAAGGG GAGCTGGGTG TGAGTGTTTA CACTGTGCCC TTCACGGGAT ACTTCTTTTA   1260
TCTGCAGATG GCCTAATGCT TAGTTGTCCA AGTCGCGATC AAGGACTCTC TCACACAGGA   1320
AACTTCCCTA TACTGGCAGA TACACTTGTG ACTGAACCAT GCCCAGTTTA TGCCTGTCTG   1380
ACTGTCACTC TGGCACTAGG AGGCTGATCT TGTACTCCAT ATGACCCCAC CCCTAGGAAC   1440
CCCCAGGGAA AACCAGGCTC GGACAGCCCC CTGTTCCTGA GATGGAAAGC ACAAATTTAA   1500
TACACCACCA CAATGGAAAA CAAGTTCAAA GACTTTTACT TACAGATCCT GGACAGAAAG   1560
GGCATAATGA GTCTGAAGGG CAGTCCTCCT TCTCCAGGTT ACATGAGGCA GGAATAAGAA   1620
GTCAGACAGA GACAGCAAGA CAGTTAACAA CGTAGGTAAA GAAATAGGGT GTGGTCACTC   1680
TCAATTCACT GGCAAATGCC TGAATGGTCT GTCTGAAGGA AGCAACAGAG AAGTGGGGAA   1740
TCCAGTCTGC TAGGCAGGAA AGATGCCTCT AAGTTCTTGT CTCTGGCCAG AGGTGTGGTA   1800
TAGAACCAGA AACCCATATC AAGGGTGACT AAGCCCGGCT TCCGGTATGA GAAATTAAAC   1860
TTGTATACAA AATGGTTGCC AAGGCAACAT AAAATTATAA GAATTC                  1906
```

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1619 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
GTCATGGAAT ACGCCTCTGA CGCTTCACTG GACCCCGAAG CCCCGTGGCC TCCCGCGCCC     60
CGCGCTCGCG CCTGCCGCGT ACTGCCTTGG GCCCTGGTCG CGGGGCTGCT GCTGCTGCTG    120
CTGCTCGCTG CCGCCTGCGC CGTCTTCCTC GCCTGCCCCT GGGCCGTGTC CGGGGCTCGC    180
GCCTCGCCCG GCTCCGCGGC CAGCCCGAGA CTCCGCGAGG GTCCCGAGCT TTCGCCCGAC    240
GATCCCGCCG GCCTCTTGGA CCTGCGGCAG GGCATGTTTG CGCAGCTGGT GGCCCAAAAT    300
GTTCTGCTGA TCGATGGGCC CCTGAGCTGG TACAGTGACC CAGGCCTGGC AGGCGTGTCC    360
CTGACGGGGG GCCTGAGCTA CAAAGAGGAC ACGAAGGAGC TGGTGGTGGC CAAGGCTGGA    420
GTCTACTATG TCTTCTTTCA ACTAGAGCTG CGGCGCGTGG TGGCCGGCGA GGGCTCAGGC    480
TCCGTTTCAC TTGCGCTGCA CCTGCAGCCA CTGCGCTCTG CTGCTGGGGC CGCCGCCCTG    540
GCTTTGACCG TGGACCTGCC ACCCGCCTCC TCCGAGGCTC GGAACTCGGC CTTCGGTTTC    600
CAGGGCCGCT TGCTGCACCT GAGTGCCGGC CAGCGCCTGG GCGTCCATCT TCACACTGAG    660
GCCAGGGCAC GCCATGCCTG GCAGCTTACC CAGGGCGCCA CAGTCTTGGG ACTCTTCCGG    720
GTGACCCCCG AAATCCCAGC CGGACTCCCT TCACCGAGGT CGGAATAACG CCCAGCCTGG    780
GTGCAGCCCA CCTGGACAGA GTCCGAATCC TACTCCATCC TTCATGGAGA CCCCTGGTGC    840
TGGGTCCCTG CTGCTTTCTC TACCTCAAGG GGCTTGGCAG GGGTCCCTGC TGCTGACCTC    900
CCCTTGAGGA CCCTCCTCAC CCACTCCTTC CCCAAGTTGG ACCTTGATAT TTATTCTGAG    960
CCTGAGCTCA GATAATATAT TATATATATT ATATATATAT ATATATTTCT ATTTAAAGAG   1020
GATCCTGAGT TTGTGAATGG ACTTTTTTAG AGGAGTTGTT TTGGGGGGGG GGTCTTCGAC   1080
ATTGCCGAGG CTGGTCTTGA ACTCCTGGAC TTAGACGATC CTCCTGCCTC AGCCTCCCAA   1140
GCAACTGGGA TTCATCCTTT CTATTAATTC ATTGTACTTA TTTGCCTATT TGTGTGTATT   1200
GAGCATCTGT AATGTGCCAG CATTGTGCCC AGGCTAGGGG GCTATAGAAA CATCTAGAAA   1260
TAGACTGAAA GAAAATCTGA GTTATGGTAA TACGTGAGGA ATTTAAAGAC TCATCCCCAG   1320
CCTCCACCTC CTGTGTGATA CTTGGGGGCT AGCTTTTTTC TTTCTTTCTT TTTTTTGAGA   1380
TGGTCTTGTT CTGTCAACCA GGCTAGAATG CAGCGGTGCA ATCATGAGTC AATGCAGCCT   1440
CCAGCCTCGA CCTCCCGAGG CTCAGGTGAT CCTCCCATCT CAGCCTCTCG AGTAGCTGGG   1500
ACCACAGTTG TGTGCCACCA CACTTGGCTA ACTTTTTAAT TTTTTTGCGG AGACGGTATT   1560
GCTATGTTGC CAAGGTTGTT TACATGCCAG TACAATTTAT AATAAACACT CATTTTTCC    1619
```

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1239 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
AGCCTATAAA GCACGGGCAC TGGCGGGAGA CGTGCACTGA CCGACCGTGG TAATGGACCA     60
GCACACACTT GATGTGGAGG ATACCGCGGA TGCCAGACAT CCAGCAGGTA CTTCGTGCCC    120
CTCGGATGCG GCGCTCCTCA GAGATACCGG GCTCCTCGCG GACGCTGCGC TCCTCTCAGA    180
TACTGTGCGC CCCACAAATG CCGCGCTCCC CACGGATGCT GCCTACCCTG CGGTTAATGT    240
TCGGGATCGC GAGGCCGCGT GGCCGCCTGC ACTGAACTTC TGTTCCCGCC ACCCAAAGCT    300
CTATGGCCTA GTCGCTTTGG TTTTGCTGCT TCTGATCGCC GCCTGTGTTC CTATCTTCAC    360
CCGCACCGAG CCTCGGCCAG CGCTCACAAT CACCACCTCG CCCAACCTGG GTACCCGAGA    420
GAATAATGCA GACCAGGTCA CCCCTGTTTC CCACATTGGC TGCCCCAACA CTACACAACA    480
GGGCTCTCCT GTGTTCGCCA AGCTACTGGC TAAAAACCAA GCATCGTTGT GCAATACAAC    540
TCTGAACTGG CACAGCCAAG ATGGAGCTGG GAGCTCATAC CTATCTCAAG GTCTGAGGTA    600
CGAAGAAGAC AAAAAGGAGT TGGTGGTAGA CAGTCCCGGG CTCTACTACG TATTTTTGGA    660
ACTGAAGCTC AGTCCAACAT TCACAAACAC AGGCCACAAG GTGCAGGGCT GGGTCTCTCT    720
TGTTTTGCAA GCAAAGCCTC AGGTAGATGA CTTTGACAAC TTGGCCCTGA CAGTGGAACT    780
GTTCCCTTGC TCCATGGAGA ACAAGTTAGT GGACCGTTCC TGGAGTCAAC TGTTGCTCCT    840
GAAGGCTGGC CACCGCCTCA GTGTGGGTCT GAGGGCTTAT CTGCATGGAG CCCAGGATGC    900
```

ATACAGAGAC TGGGAGCTGT CTTATCCCAA CACCACCAGC TTTGGACTCT TTCTTGTGAA 960
ACCCGACAAC CCATGGGAAT GAGAACTATC CTTCTTGTGA CTCCTAGTTG CTAAGTCCTC 1020
AAGCTGCTAT GTTTTATGGG GTCTGAGCAG GGGTCCCTTC CATGACTTTC TCTTGTCTTT 1080
AACTGGACTT GGTATTTATT CTGAGCATAG CTCAGACAAG ACTTTATATA ATTCACTAGA 1140
TAGCATTAGT AAACTGCTGG GCAGCTGCTA GATAAAAAAA AATTTCTAAA TCAAAGTTTA 1200
TATTTATATT AATATATAAA AATAAATGTG TTTGTAAAT 1239

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 606 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ATGATCGAAA CATACAACCA AACTTCTCCC CGATCTGCGG CCACTGGACT GCCCATCAGC 60
ATGAAAATTT TTATGTATTT ACTTACTGTT TTTCTTATCA CCCAGATGAT TGGGTCAGCA 120
CTTTTTGCTG TGTATCGCTT CGCACAGGCT TTTGAAATGC AAAAAGGTGA TCAGAATCCT 180
CAAATTGCGG CACATGTCAT AAGTGAGGCC AGCAGTAAAA CAACATCTGT GTTACAGTGG 240
GCTGAAAAAG GATACTACAC CATGAGCAAC AACTTGGTAA CCCTGGAAAA TGGGAAACAG 300
CTGACCGTTA AAAGACAAGG ACTCTATTAT ATCTATGCCC AAGTCACCTT CTGTTCCAAT 360
CGGGAAGCTT CGAGTCAAGC TCCATTTATA GCCAGCCTCT GCCTAAAGTC CCCCGGTAGA 420
TTCGAGAGAA TCTTACTCAG AGCTGCAAAT ACCCACAGTT CCGCCAAACC TTGCGGGCAA 480
CAATCCATTC ACTTGGGAGG AGTATTTGAA TTGCAACCAG GTGCTTCGGT GTTTGTCAAT 540
GTGACTGATC CAAGCCAAGT GAGCCATGGC ACTGGCTTCA CGTCCTTTGG CTTACTCAAA 600
CTCTGA 606

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 783 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ATGATCGAAA CATACAACCA AACTTCTCCC CGATCTGCGG CCACTGGACT GCCCATCAGC 60
ATGAAAATTT TTATGTATTT ACTTACTGTT TTTCTTATCA CCCAGATGAT TGGGTCAGCA 120
CTTTTTGCTG TGTATCTTCA TAGAAGATTG GATAAGGTCG AAGAGGAAGT AAACCTTCAT 180
GAAGATTTTG TATTCATAAA AAAGCTAAAG AGATGCAACA AAGGAGAAGG ATCTTTATCC 240
TTGCTGAACT GTGAGGAGAT GAGAAGGCAA TTTGAAGACC TTGTCAAGGA TATAACGTTA 300
AACAAAGAAG AGAAAAAAGA AAACAGCTTT GAAATGCAAA AAGGTGATCA GAATCCTCAA 360
ATTGCGGCAC ATGTCATAAG TGAGGCCAGC AGTAAAACAA CATCTGTGTT ACAGTGGGCT 420
GAAAAAGGAT ACTACACCAT GAGCAACAAC TTGGTAACCC TGGAAAATGG GAAACAGCTG 480
ACCGTTAAAA GACAAGGACT CTATTATATC TATGCCCAAG TCACCTTCTG TTCCAATCGG 540
GAAGCTTCGA GTCAAGCTCC ATTTATAGCC AGCCTCTGCC TAAAGTCCCC CGGTAGATTC 600
GAGAGAATCT TACTCAGAGC TGCAAATACC CACAGTTCCG CCAAACCTTG CGGGCAACAA 660
TCCATTCACT TGGGAGGAGT ATTTGAATTG CAACCAGGTG CTTCGGTGTT TGTCAATGTG 720
ACTGATCCAA GCCAAGTGAG CCATGGCACT GGCTTCACGT CCTTTGGCTT ACTCAAACTC 780
TGA 783

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 558 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

```
CTGCTGCACT TCGGGGTAAT CGGCCCCCAG AGGGAAGAGC AGTCCCCAGG TGGCCCCTCC      60
ATCAACAGCC CTCTGGTTCA AACACTCAGG TCCTCTTCTC AAGCCTCAAG TAACAAGCCG     120
GTAGCCCACG TTGTAGCCGA CATCAACTCT CCGGGGCAGC TCCGGTGGTG GGACTCGTAT     180
GCCAATGCCC TCATGGCCAA CGGTGTGAAG CTGGAAGACA ACCAGCTGGT GGTGCCTGCT     240
GACGGGCTTT ACCTCATCTA CTCACAGGTC CTCTTCAGGG GCCAAGGCTG CCCTTCCACC     300
CCCTTGTTCC TCACCCACAC CATCAGCCGC ATTGCAGTCT CCTACCAGAC CAAGGTCAAC     360
ATCCTGTCTG CCATCAAGAG CCCTTGCCAC AGGGAGACCC CAGAGTGGGC TGAGGCCAAG     420
CCCTGGTACG AACCCATCTA CCAGGGAGGA GTCTTCCAGC TGGAGAAGGG AGATCGCCTC     480
AGTGCTGAGA TCAACCTGCC GGACTACCTG GACTATGCCG AGTCCGGGCA GGTCTACTTT     540
GGGATCATTG CCCTGTGA                                                   558
```

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1783 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

```
CAAGTCACAT GATCCAGGAT GCAGGGGAAA ATCCTTCTTG GAACAGAGCT GGGTACAGAA      60
CCGAATCAGA TGAGGAGAGA TAAGGTGTGA TGTGGGACAG ACTATATAAA GCATGGAGCC     120
AGGGCTGCAA CAAGCAGGCA GCTGTGGGGC TCCTTCCCCT GACCCAGCCA TGCAGGTGCA     180
GCCCGGCTCG GTAGCCAGCC CCTGGAGAAG CACGAGGCCC TGGAGAAGCA CAAGTCGCAG     240
CTACTTCTAC CTCAGCACCA CCGCACTGGT GTGCCTTGTT GTGGCAGTGG CGATCATTCT     300
GGTACTGGTA GTCCAGAAAA AGGACTCCAC TCCAAATACA ACTGAGAAGG CCCCCCTTAA     360
AGGAGGAAAT TGCTCAGAGG ATCTCTTCTG TACCCTGAAA AGTACTCCAT CCAAGAAGTC     420
ATGGGCCTAC CTCCAAGTGT CAAAGCATCT CAACAATACC AAACTGTCAT GGAACGAAGA     480
TGGCACCATC CACGGACTCA TATACCAGGA CGGGAACCTG ATAGTCCAAT TCCCTGGCTT     540
GTACTTCATC GTTTGCCAAC TGCAGTTCCT CGTGCAGTGC TCAAATCATT CTGTGGACCT     600
GACATTGCAG CTCCTCATCA ATTCCAAGAT CAAAAAGCAG ACGTTGGTAA CAGTGTGTGA     660
GTCTGGAGTT CAGAGTAAGA ACATCTACCA GAATCTCTCT CAGTTTTTGC TGCATTACTT     720
ACAGGTCAAC TCTACCATAT CAGTCAGGGT GGATAATTTC CAGTATGTGG ATACAAACAC     780
TTTCCCTCTT GATAATGTGC TATCCGTCTT CTTATATAGT AGCTCAGACT GAATAGTTGT     840
TCTTAACCTT TATGAAAATG CTGTCTACCA TACAGTACTT CATCTGTCCA AACATGGGCC     900
AAAGAAAATA TTAGGACAAC TCAAACTAAG CATGTGAGTT AGTGCACTTC TCTTTCTGTC     960
CTTTGGAAAA ATACAAACCC AGGATTTAGA AAGTGGAGTC TCCTTCAGAT GCACAAACAG    1020
GAAAGAATGT GATATGTGCA CAGAGACCTA CTTGGGCACT AGAAGGGGTG TGAGTTGTCC    1080
CAGTATAACC ACTAATTCAC TGACCTTGAG CCATTTTTCC TTCCCCCTGG AACTTGGGGT    1140
CTGAATCTGG AAAAGTAGGA GATGAGATTT ACATTTCCCC AATATTTTCT TCAACTCAGA    1200
AGACGAGACT GTGGAGCTGA GCTCCCTACA CAGATGAAGG CCTCCCATGG CATGAGGAAA    1260
ATGATGGTAC CAGTAATGTC TGTCTGACTG TCATCTCAGC AAGTCCTAAG GACTTCCATG    1320
CTGCCTTGTT GAAAGATACT CTAACCTCTT GTAATGGGCA AAGTGATCCT GTCTCTCACT    1380
GAGGGGAGTA GCTGCTGCCA TCTCCTGAGA CATACATGGA GACATTTTCT GCCCAAATTC    1440
CATTCTGTGT GCAGTTTTTA AGTATTCCCC CAAAAGTTCT TGACAATGAG AACTTTGAAT    1500
GTGGGAAGAG CTTCTGGACA GCAAACATTA ACAGCTTCTC CTGACCAGAG AGACCATGCA    1560
AGCTTGGTCT TAGACCCATC AAGCTTGAGG TTTCTACATT GTGGGAGACA GACTTTTGAC    1620
AAACCATTTG AGTTGATGTC TGGGCCCCTG GGAGTTCTCC TTCAGTAAGG AGAGCAAGCC    1680
GTTCTAGTGC TGTGTCAGAG GATGGAGTAA AATAGACACT TTTCTGAAGG AAAGGAGAAC    1740
AAAGTTCCAG AAAAAGGCTA GAAAATGTTT AAAAAGAAAA AAA                      1783
```

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1047 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

```
AGAGAGCGCT GGGAGCCGGA GGGGAGCGCA GCGAGTTTTG GCCAGTGGTC GTGCAGTCCA     60
AGGGGCTGGA TGGCATGCTG GACCCAAGCT CAGCTCAGCG TCCGGACCCA ATAACAGTTT    120
TACCAAGGGA GCAGCTTTCT ATCCTGGCCA CACTGAGGTG CATAGCGTAA TGTCCATGTT    180
GTTCTACACT CTGATCACAG CTTTTCTGAT CGGCATACAG GCGGAACCAC ACTCAGAGAG    240
CAATGTCCCT GCAGGACACA CCATCCCCCA AGTCCACTGG ACTAAACTTC AGCATTCCCT    300
TGACACTGCC CTTCGCAGAG CCCGCAGCGC CCCGGCAGCG GCGATAGCTG CACGCGTGGC    360
GGGGCAGACC CGCAACATTA CTGTGGACCC CAGGCTGTTT AAAAAGCGGC GACTCCGTTC    420
ACCCCGTGTG CTGTTTAGCA CCCAGCCTCC CCGTGAAGCT GCAGACACTC AGGATCTGGA    480
CTTCGAGGTC GGTGGTGCTG CCCCCTTCAA CAGGACTCAC AGGAGCAAGC GGTCATCATC    540
CCATCCCATC TTCCACAGGG GCGAATTCTC GGTGTGTGAC AGTGTCAGCG TGTGGGTTGG    600
GGATAAGACC ACCGCCACAG ACATCAAGGG CAAGGAGGTG ATGGTGTTGG GAGAGGTGAA    660
CATTAACAAC AGTGTATTCA AACAGTACTT TTTTGAGACC AAGTGCCGGG ACCCAAATCC    720
CGTTGACAGC GGGTGCCGGG GCATTGACTC AAAGCACTGG AACTCATATT GTACCACGAC    780
TCACACCTTT GTCAAGGCGC TGACCATGGA TGGCAAGCAG GCTGCCTGGC GGTTTATCCG    840
GATAGATACG GCCTGTGTGT GTGTGCTCAG CAGGAAGGCT GTGAGAAGAG CCTGACCTGC    900
CGACACGCTC CCTCCCCCTG CCCCTTCTAC ACTCTCCTGG GCCCCTCCCT ACCTCAACCT    960
GTAAATTATT TTAAATTATA AGGACTGCAT GGTAATTTAT AGTTTATACA GTTTTAAAGA   1020
ATCATTATTT ATTAAATTTT TGGAAGC                                       1047
```

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1176 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

```
GAGCGCCTGG AGCCGGAGGG GAGCGCATCG AGTGACTTTG GAGCTGGCCT TATATTTGGA     60
TCTCCCGGGC AGCTTTTTGG AAACTCCTAG TGAACATGCT GTGCCTCAAG CCAGTGAAAT    120
TAGGCTCCCT GGAGGTGGGA CACGGGCAGC ATGGTGGAGT TTTGGCCTGT GGTCGTGCAG    180
TCCAGGGGGC TGGATGGCAT GCTGGACCCA AGCTCACCTC AGTGTCTGGG CCCAATAAAG    240
GTTTTGCCAA GGACGCAGCT TTCTATACTG GCCGCAGTGA GGTGCATAGC GTAATGTCCA    300
TGTTGTTCTA CACTCTGATC ACTGCGTTTT TGATCGGCGT ACAGGCAGAA CCGTACACAG    360
ATAGCAATGT CCCAGAAGGA GACTCTGTCC CTGAAGCCCA CTGGACTAAA CTTCAGCATT    420
CCCTTGACAC AGCCCTCCGC AGAGCCCGCA GTGCCCCTAC TGCACCAATA GCTGCCCGAG    480
TGACAGGGCA GACCCGCAAC ATCACTGTAG ACCCCAGACT GTTTAAGAAA CGGAGACTCC    540
ACTCACCCCG TGTGCTGTTC AGCACCCAGC CTCCACCCAC CTCTTCAGAC ACTCTGGATC    600
TAGACTTCCA GGCCCATGGT ACAATCCCTT TCAACAGGAC TCACCGGAGC AAGCGCTCAT    660
CCACCCACCC AGTCTTCCAC ATGGGGGAGT TCTCAGTGTG TGACAGTGTC AGTGTGTGGG    720
TTGGAGATAA GACCACAGCC ACAGACATCA AGGGCAAGGA GGTGACAGTG CTGGCCGAGG    780
TGAACATTAA CAACAGTGTA TTCAGACAGT ACTTTTTTGA GACCAAGTGC CGAGCCTCCA    840
ATCCTGTTGA GAGTGGGTGC CGGGGCATCG ACTCCAAACA CTGGAACTCA TACTGCACCA    900
CGACTCACAC CTTCGTCAAG GCGTTGACAA CAGATGAGAA GCAGGCTGCC TGGAGGTTCA    960
TCCGGATAGA CACAGCCTGT GTGTGTGTGC TCAGCAGGAA GGCTACAAGA AGAGGCTGAC   1020
TTGCCTGCAG CCCCCTTCCC CACCTGCCCC CTCCACACTC TCTTGGGCCC CTCCCTACCT   1080
CAGCCTGTAA ATTATTTTAA ATTATAAGGA CTGCATGATA ATTTATCGTT TATACAATTT   1140
TAAAGACATT ATTTATTAAA TTTTCAAAGC ATCCTG                              1176
```

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1623 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
TCAGAGTCCT GTCCTTGACA CTTCAGTCTC CACAAGACTG AGAGGAGGAA ACCCTTTCCT     60
GGGGCTGGGT GCCATGCAGC AGCCCGTGAA TTACCCATGT CCCCAGATCT ACTGGGTAGA    120
CAGCAGTGCC ACTTCTCCTT GGGCTCCTCC AGGGTCAGTT TTTTCTTGTC CATCCTCTGG    180
GCCTAGAGGG CCAGGACAAA GGAGACCACC GCCTCCACCA CCACCTCCAT CACCACTACC    240
ACCGCCTTCC CAACCACCCC CGCTGCCTCC ACTAAGCCCT CTAAAGAAGA AGGACAACAT    300
AGAGCTGTGG CTACCGGTGA TATTTTTCAT GGTGCTGGTG GCTCTGGTTG GAATGGGGTT    360
AGGAATGTAT CAACTCTTTC ATCTACAGAA GGAACTGGCA GAACTCCGTG AGTTCACCAA    420
CCACAGCCTT AGAGTATCAT CTTTTGAAAA GCAAATAGCC AACCCCAGCA CACCCTCTGA    480
AACCAAAAAG CCAAGGAGTG TGGCCCACTT AACAGGGAAC CCCCGCTCAA GGTCCATCCC    540
TCTGGAATGG GAAGACACAT ATGGAACTGC TTTGATCTCT GGAGTGAAGT ATAAGAAAGG    600
CGGCCTTGTG ATCAATGAGG CTGGGTTGTA CTTCGTATAT TCCAAAGTAT ACTTCCGGGG    660
TCAGTCTTGC AACAGCCAGC CCCTAAGCCA CAAGGTCTAT ATGAGGAACT TTAAGTATCC    720
TGGGGATCTG GTGCTAATGG AGGAGAAGAA GTTGAATTAC TGCACTACTG GCCAGATATG    780
GGCCCACAGC AGCTACCTAG GGGCAGTATT TAATCTTACC GTTGCTGACC ATTTATATGT    840
CAACATATCT CAACTCTCTC TGATCAATTT TGAGGAATCT AAGACCTTTT TTGGCTTATA    900
TAAGCTTTAA AGGAAAAAGC ATTTTAGAAT GATCTATTAT TCTTTATCAT GGATGCCAGG    960
AATATTGTCT TCAATGAGAG TCTTCTTAAG ACCAATTGAG CCACAAAGAC CACAAGGTCC   1020
AACAGGTCAG CTACCCTTCA TTTTCTAGAG GTCCATGGAG TGGTCCTTAA TGCCTGCATC   1080
ATGAGCCAGA TGGGAAGAAG ACTGTTCCTG AGGAACATAA AGTTTTGGGC TGCTGTGTGG   1140
CAATGCAGAG GCAAAGAGAA GGAACTGTCT GATGTTAAAT GGCCAAGAGC ATTTTAGCCA   1200
TTGAAGAAAA AAAAAACCTT TAAACTCACC TTCCAGGGTG GGTCTACTTG CTACCTCACA   1260
GGAGGCCGTC TTTTAGACAC ATGGTTGTGG TATGACTATA CAAGGGTGAG AAAGGATGCT   1320
AGGTTTCATG GATAAGCTAG AGACTGAAAA AAGCCAGTGT CCCATTGGCA TCATCTTTAT   1380
TTTTAACTGA TGTTTTCTGA GCCCACCTTT GATGCTAACA GAGAAATAAG AGGGGTGTTT   1440
GAGGCACAAG TCATTCTCTA CATAGCATGT GTACCTCCAG TGCAATGATG TCTGTGTGTG   1500
TTTTTATGTA TGAGAGTAGA GCGATTCTAA AGAGTCACAT GAGTACAACG CGTACATTAC   1560
GGAGTACATA TTAGAAACGT ATGTGTTACA TTTGATGCTA GAATATCTGA ATGTTTCTTG   1620
CTA                                                                 1623
```

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

```
GTTAAGCTTT TCAGTCAGCA TGATAGAA                                        28
```

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

```
GTTTCTAGAT CAGAGTTTGA GTAAGCC                                         27
```

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CCAAGACTAG TTAACACAGC ATGATCGAAA 30

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CCAATGCGGC CGCACTCAGA ATTCAACCTG 30

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 972 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

```
TCTAGACTCA GGACTGAGAA GAAGTAAAAC CGTTTGCTGG GGCTGGCCTG ACTCACCAGC    60
TGCCATGCAG CAGCCCTTCA ATTACCCATA TCCCCAGATC TACTGGGTGG ACAGCAGTGC   120
CAGCTCTCCC TGGGCCCCTC CAGGCACAGT TCTTCCCTGT CCAACCTCTG TGCCCAGAAG   180
GCCTGGTCAA AGGAGGCCAC CACCACCACC GCCACCGCCA CCACTACCAC CTCCGCCGCC   240
GCCGCCACCA CTGCCTCCAC TACCGCTGCC ACCCCTGAAG AAGAGAGGGA ACCACAGCAC   300
AGGCCTGTGT CTCCTTGTGA TGTTTTTCAT GGTTCTGGTT GCCTTGGTAG GATTGGGCCT   360
GGGGATGTTT CAGCTCTTCC ACCTACAGAA GGAGCTGGCA GAACTCCGAG AGTCTACCAG   420
CCAGATGCAC ACAGCATCAT CTTTGGAGAA GCAAATAGGC CACCCCAGTC CACCCCCCTGA  480
AAAAAAGGAG CTGAGGAAAG TGGCCCATTT AACAGGCAAG TCCAACTCAA GGTCCATGCC   540
TCTGGAATGG GAAGACACCT ATGGAATTGT CCTGCTTTCT GGAGTGAAGT ATAAGAAGGG   600
TGGCCTTGTG ATCAATGAAA CTGGGCTGTA CTTTGTATAT TCCAAAGTAT ACTTCCGGGG   660
TCAATCTTGC AACAACCTGC CCCTGAGCCA CAAGGTCTAC ATGAGGAACT CTAAGTATCC   720
CCAGGATCTG GTGATGATGG AGGGGAAGAT GATGAGCTAC TGCACTACTG GGCAGATGTG   780
GGCCCGCAGC AGCTACCTGG GGGCAGTGTT CAATCTTACC AGTGCTGATC ATTTATATGT   840
CAACGTATCT GAGCTCTCTC TGGTCAATTT TGAGGAATCT CAGACGTTTT TCGGCTTATA   900
TAAGCTCTAA GAGAAGCACT TTGGGATTCT TTCCATTATG ATTCTTTGTT ACAGGCACCG   960
AGATGTTCTA GA                                                       972
```

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 885 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

```
ATGCAGCAGC CCATGAATTA CCCATGTCCC CAGATCTTCT GGGTAGACAG CAGTGCCACT    60
TCATCTTGGG CTCCTCCAGG GTCAGTTTTT CCCTGTCCAT CTTGTGGGCC TAGAGGGCCG   120
GACCAAAGGA GACCGCCACC TCCACCACCA CCTGTGTCAC CACTACCACC GCCATCACAA   180
CCACTCCCAC TGCCGCCACT GACCCCTCTA AAGAAGAAGG ACCACAACAC AAATCTGTGG   240
CTACCGGTGG TATTTTTCAT GGTTCTGGTG GCTCTGGTTG GAATGGGATT AGGAATGTAT   300
CAGCTCTTCC ACCTGCAGAA GGAACTGGCA GAACTCCGTG AGTTCACCAA CCAAAGCCTT   360
AAAGTATCAT CTTTTGAAAA GCAAATAGCC AACCCCAGTA CACCCTCTGA AAAAAAAGAG   420
CCGAGGAGTG TGGCCCATTT AACAGGGAAC CCCCACTCAA GGTCCATCCC TCTGGAATGG   480
GAAGACACAT ATGGAACCGC TCTGATCTCT GGAGTGAAGT ATAAGAAAGG TGGCCTTGTG   540
ATCAACGAAG CTGGGTTGTA CTTCGTATAT TCCAAAGTAT ACTTCCGGGG TCAGTCTTGC   600
AACAACCAGC CCCTAAACCA CAAGGTCTAT ATGAGGAACT CTAAGTATCC TGGGGATCTG   660
GTGCTAATGG AGGAGAAGAG GTTGAACTAC TGCACTACTG GACAGATATG GGCCCACAGC   720
AGCTACCTGG GGGCAGTATT CAATCTTACC AGTGCTGACC ATTTATATGT CAACATATCT   780
CAACTCTCTC TGATCAATTT TGAGGAATCT AAGACCTTTT TCGGCTTGTA TAAGCTTTAA   840
AAGAAAAAGC ATTTTAAAAT GATCTACTAT TCTTTATCAT GGGCA                    885
```

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

```
CTTAAGCTTC TACAGGACTG AGAAGAAGT    29
```

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

```
CTTGAATTCC AACATTCTCG GTGCCTGTAA    27
```

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

```
TCAGGATCCA CAAGGCTGTG AGAAGGA    27
```

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CTTGTCTAGA CCTGGTGCC CATGATA 27

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 680 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

ATGCCGGAGG AAGGTCGCCC TTGCCCCTGG GTTCGCTGGA GCGGGACCGC GTTCCAGCGC 60
CAATGGCCAT GGCTGCTGCT GGTGGTGTTT ATTACTGTGT TTTGCTGTTG GTTTCATTGT 120
AGCGGACTAC TCAGTAAGCA GCAACAGAGG CTGCTGGAGC ACCCTGAGCC GCACACAGCT 180
GAGTTACAGC TGAATCTCAC AGTTCCTCGG AAGGACCCCA CACTGCGCTG GGGAGCAGGC 240
CCAGCCTTGG GAAGGTCCTT CACACACGGA CCAGAGCTGG AGGAGGGCCA TCTGCGTATC 300
CATCAAGATG GCCTCTACAG GCTGCATATC CAGGTGACAC TGGCCAACTG CTCTTCCCCA 360
GGCAGCACCC TGCAGCACAG GGCCACCCTG GCTGTGGGCA TCTGCTCCCC CGCTGCGCAC 420
GGCATCAGCT TGCTGCGTGG GCGCTTTGGA CAGGACTGTA CAGTGGCATT ACAGCGCCTG 480
ACATACCTGG TCCACGGAGA TGTCCTCTGT ACCAACCTCA CCCTGCCTCT GCTGCCGTCC 540
CGCAACGCTG ATGAGACCTT CTTTGGAGTT CAGTGGATAT GCCCTTGACC ACAACTCCAG 600
GATGACTTGT GAATATTTTT TTTCTTTTCA AGTTCTACGT ATTTATAAAT GTATATAGTA 660
CACATAAAAA AAAAAAAAAA 680

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 846 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

ATGCAGCAGC CCTTCAATTA CCCATATCCC CAGATCTACT GGGTGGACAG CAGTGCCAGC 60
TCTCCCTGGG CCCCTCCAGG CACAGTTCTT CCCTGTCCAA CCTCTGTGCC CAGAAGGCCT 120
GGTCAAAGGA GGCCACCACC ACCACCGCCA CCGCCACCAC TACCACCTCC GCCGCCGCCG 180
CCACCACTGC CTCCACTACC GCTGCCACCC CTGAAGAAGA GAGGGAACCA CAGCACAGGC 240
CTGTGTCTCC TTGTGATGTT TTTCATGGTT CTGGTTGCCT TGGTAGGATT GGGCCTGGGG 300
ATGTTTCAGC TCTTCCACCT GCAGAAGGAA CTGGCAGAAC TCCGTGAGTT CACCAACCAA 360
AGCCTTAAAG TATCATCTTT TGAAAAGCAA ATAGGCCACC CCAGTCCACC CCCTGAAAAA 420
AAGGAGCTGA GGAAAGTGGC CCATTTAACA GGCAAGTCCA ACTCAAGGTC CATGCCTCTG 480
GAATGGGAAG ACACCTATGG AATTGTCCTG CTTTCTGGAG TGAAGTATAA GAAGGGTGGC 540
CTTGTGATCA ATGAAACTGG GCTGTACTTT GTATATTCCA AAGTATACTT CCGGGGTCAA 600
TCTTGCAACA ACCTGCCCCT GAGCCACAAG GTCTACATGA GGAACTCTAA GTATCCCCAG 660
GATCTGGTGA TGATGGAGGG GAAGATGATG AGCTACTGCA CTACTGGGCA GATGTGGGCC 720
CGCAGCAGCT ACCTGGGGGC AGTGTTCAAT CTTACCAGTG CTGATCATTT ATATGTCAAC 780

GTATCTGAGC TCTCTCTGGT CAATTTTGAG GAATCTCAGA CGTTTTTCGG CTTATATAAG 840
CTCTAA 846

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 786 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

ATGCAGCAGC CCTTCAATTA CCCATATCCC CAGATCTACT GGGTGGACAG CAGTGCCAGC 60
TCTCCCTGGG CCCCTCCAGG CACAGTTCTT CCCTGTCCAA CCTCTGTGCC CAGAAGGCCT 120
GGTCAAAGGA GGCCACCACC ACCACCGCCA CCGCCACCAC TACCACCTCC GCCGCCGCCG 180
CCACCACTGC CTCCACTACC GCTGCCACCC CTGAAGAAGA GAGGGAACCA CAGCACAGGC 240
CTGTGTCTCC TTGTGATGTT TTTCATGGTT CTGGTTGCCT TGGTAGGATT GGGCCTGGGG 300
ATGTTTCAGC TCTTCCGCTT CGCACAGGCT ATAGGCCACC CCAGTCCACC CCCTGAAAAA 360
AAGGAGCTGA GGAAAGTGGC CCATTTAACA GGCAAGTCCA ACTCAAGGTC CATGCCTCTG 420
GAATGGGAAG ACACCTATGG AATTGTCCTG CTTTCTGGAG TGAAGTATAA GAAGGGTGGC 480
CTTGTGATCA ATGAAACTGG GCTGTACTTT GTATATTCCA AAGTATACTT CCGGGGTCAA 540
TCTTGCAACA ACCTGCCCCT GAGCCACAAG GTCTACATGA GGAACTCTAA GTATCCCCAG 600
GATCTGGTGA TGATGGAGGG GAAGATGATG AGCTACTGCA CTACTGGGCA GATGTGGGCC 660
CGCAGCAGCT ACCTGGGGGC AGTGTTCAAT CTTACCAGTG CTGATCATTT ATATGTCAAC 720
GTATCTGAGC TCTCTCTGGT CAATTTTGAG GAATCTCAGA CGTTTTTCGG CTTATATAAG 780
CTCTAA 786

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 864 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

ATGCAGCAGC CCTTCAATTA CCCATATCCC CAGATCTACT GGGTGGACAG CAGTGCCAGC 60
TCTCCCTGGG CCCCTCCAGG CACAGTTCTT CCCTGTCCAA CCTCTGTGCC CAGAAGGCCT 120
GGTCAAAGGA GGCCACCACC ACCACCGCCA CCGCCACCAC TACCACCTCC GCCGCCGCCG 180
CCACCACTGC CTCCACTACC GCTGCCACCC CTGAAGAAGA GAGGGAACCA CAGCACAGGC 240
CTGTGTCTCC TTGTGATGTT TTTCATGGTT CTGGTTGCCT TGGTAGGATT GGGCCTGGGG 300
ATGTTTCAGC TCTTCCAATC CTCCATCCTC CCCTATGCCG GAGGAGGGTT CGGGCTGCTC 360
GGTGCGGCGC AGGCCCTATG GGTGCGTCCT GCGGCCATCC TCAATCCTAT AGGCCACCCC 420
AGTCCACCCC CTGAAAAAAA GGAGCTGAGG AAAGTGGCCC ATTTAACAGG CAAGTCCAAC 480
TCAAGGTCCA TGCCTCTGGA ATGGGAAGAC ACCTATGGAA TTGTCCTGCT TTCTGGAGTG 540
AAGTATAAGA AGGGTGGCCT TGTGATCAAT GAAACTGGGC TGTACTTTGT ATATTCCAAA 600
GTATACTTCC GGGGTCAATC TTGCAACAAC CTGCCCCTGA GCCACAAGGT CTACATGAGG 660
AACTCTAAGT ATCCCCAGGA TCTGGTGATG ATGGAGGGGA AGATGATGAG CTACTGCACT 720
ACTGGGCAGA TGTGGGCCCG CAGCAGCTAC CTGGGGGCAG TGTTCAATCT TACCAGTGCT 780
GATCATTTAT ATGTCAACGT ATCTGAGCTC TCTCTGGTCA ATTTTGAGGA ATCTCAGACG 840
TTTTTCGGCT TATATAAGCT CTAA
864

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 828 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

```
ATGCAGCAGC CCTTCAATTA CCCATATCCC CAGATCTACT GGGTGGACAG CAGTGCCAGC     60
TCTCCCTGGG CCCCTCCAGG CACAGTTCTT CCCTGTCCAA CCTCTGTGCC CAGAAGGCCT    120
GGTCAAAGGA GGCCACCACC ACCACCGCCA CCGCCACCAC TACCACCTCC GCCGCCGCCG    180
CCACCACTGC CTCCACTACC GCTGCCACCC CTGAAGAAGA GAGGGAACCA CAGCACAGGC    240
CTGTGTCTCC TTGTGATGTT TTTCATGGTT CTGGTTGCCT TGGTAGGATT GGGCCTGGGG    300
ATGTTTCAGC TCTTCCACCT ACAGCGAGAG TCTACCAGCC AGATGCACAC AGCATCATCT    360
TTGGAGAAGC AAATAGGCCA CCCCAGTCCA CCCCCTGAAA AAAAGGAGCT GAGGAAAGTG    420
GCCCATTTAA CAGGCAAGTC CAACTCAAGG TCCATGCCTC TGGAATGGGA AGACACCTAT    480
GGAATTGTCC TGCTTTCTGG AGTGAAGTAT AAGAAGGGTG GCCTTGTGAT CAATGAAACT    540
GGGCTGTACT TTGTATATTC CAAAGTATAC TTCCGGGGTC AATCTTGCAA CAACCTGCCC    600
CTGAGCCACA AGGTCTACAT GAGGAACTCT AAGTATCCCC AGGATCTGGT GATGATGGAG    660
GGGAAGATGA TGAGCTACTG CACTACTGGG CAGATGTGGG CCCGCAGCAG CTACCTGGGG    720
GCAGTGTTCA ATCTTACCAG TGCTGATCAT TTATATGTCA ACGTATCTGA GCTCTCTCTG    780
GTCAATTTTG AGGAATCTCA GACGTTTTTC GGCTTATATA AGCTCTAA                 828
```

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 846 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

```
ATGGCTATGA TGGAGGTCCA GGGGGGACCC AGCCTGGGAC AGACCTGCGT GCTGATCGTG     60
ATCTTCACAG TGCTCCTGCA GTCTCTCTGT GTGGCTGTAA CTTACGTGTA CTTTACCAAC    120
GAGCTGAAGC AGATGCAGGA CAAGTACTCC AAAAGTGGCA TTGCTTGTTT CTTAAAAGAA    180
GATGACAGTT ATTGGGACCC CAATGACGAA GAGAGTATGA ACAGCCCCTG CTGGCAAGTC    240
AAGTGGCAAC TCCGTCAGCT CGTTAGAAAG ATGATTTTGA GAACCTCTGA GGAAACCATT    300
TCTACAGTTC AAGAAAAGCA ACAAAATATT TCTCCCCTAG TGAGAGAAAG AGGTCCTCAG    360
AGAGTAGCAG CTCACATAAC TGGGACCAGA GGAAGAAGCA ACACATTGTC TTCTCCAAAC    420
TCCAAGAATG AAAAGGCTCT GGGCCGCAAA ATAAACTCCT GGGAATCATC AAGGAGTGGG    480
CATTCATTCC TGAGCAACTT GCACTTGAGG AATGGTGAAC TGGTCATCCA TGAAAAAGGG    540
TTTTACTACA TCTATTCCCA AACATACTTT CGATTTCAGG AGGAAATAAA AGAAAACACA    600
AAGAACGACA AACAAATGGT CCAATATATT TACAAATACA CAAGTTATCC TGACCCTATA    660
TTGTTGATGA AAAGTGCTAG AAATAGTTGT TGGTCTAAAG ATGCAGAATA TGGACTCTAT    720
TCCATCTATC AAGGGGGAAT ATTTGAGCTT AAGGAAAATG ACAGAATTTT TGTTTCTGTA    780
ACAAATGAGC ACTTGATAGA CATGGACCAT GAAGCCAGTT TTTTCGGGGC CTTTTTAGTT    840
GGCTAA                                                               846
```

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 876 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

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ATGCCTTCCT CAGGGGCCCT GAAGGACCTC AGCTTCAGTC AGCACTTCAG GATGATGGTG     60
ATTTGCATAG TGCTCCTGCA GGTGCTCCTG CAGGCTGTGT CTGTGGCTGT GACTTACATG    120
```

```
TACTTCACCA ACGAGATGAA GCAGCTGCAG GACAATTACT CCAAAATTGG ACTAGCTTGC     180
TTCTCAAAGA CGGATGAGGA TTTCTGGGAC TCCACTGATG GAGAGATCTT GAACAGACCC     240
TGCTTGCAGG TTAAGAGGCA ACTGTATCAG CTCATTGAAG AGGTGACTTT GAGAACCTTT     300
CAGGACACCA TTTCTACAGT TCCAGAAAAG CAGCTAAGTA CTCCTCCCTT GCCCAGAGGT     360
GGAAGACCTC AGAAAGTGGC AGCTCACATT ACTGGGATCA CTCGGAGAAG CAACTCAGCT     420
TTAATTCCAA TCTCCAAGGA TGGAAAGACC TTAGGCCAGA AGATTGAATC CTGGGAGTCC     480
TCTCGGAAAG GGCATTCATT TCTCAACCAC GTGCTCTTTA GGAATGGAGA GCTGGTCATC     540
GAGCAGGAGG GCCTGTATTA CATCTATTCC CAAACATACT TCCGATTTCA GGAAGCTGAA     600
GACGCTTCCA AGATGGTCTC AAAGGACAAG GTGAGAACCA AACAGCTGGT GCAGTACATC     660
TACAAGTACA CCAGCTATCC GGATCCCATA GTGCTCATGA AGAGCGCCAG AAACAGCTGT     720
TGGTCCAGAG ATGCCGAGTA CGGACTGTAC TCCATCTATC AGGGAGGATT GTTCGAGCTA     780
AAAAAAAATG ACAGGATTTT TGTTTCTGTG ACAAATGAAC ATTTGATGGA CCTGGATCAA     840
GAAGCCAGCT TCTTTGGAGC CTTTTTAATT AACTAA                               876
```

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 720 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

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CAGGTGCAGC CCGGCTCGGT AGCCAGCCCC TGGAGAAGCA CGAGGCCCTG GAGAAGCACA     120
AGTCGCAGCT ACTTCTACCT CAGCACCACC GCACTGGTGT GCCTTGTTGT GGCAGTGGCG     180
ATCATTCTGG TACTGGTAGT CCAGAAAAAG GACTCCACTC CAAATACAAC TGAGAAGGCC     240
CCCCTTAAAG GAGGAAATTG CTCAGAGGAT CTCTTCTGTA CCCTGAAAAG TACTCCATCC     300
AAGAAGTCAT GGGCCTACCT CCAAGTGTCA AAGCATCTCA ACAATACCAA ACTGTCATGG     360
AACGAAGATG GCACCATCCA CGGACTCATA TACCAGGACG GGAACCTGAT AGTCCAATTC     420
CCTGGCTTGT ACTTCATCGT TTGCCAACTG CAGTTCCTCG TGCAGTGCTC AAATCATTCT     480
GTGGACCTGA CATTGCAGCT CCTCATCAAT TCCAAGATCA AAAAGCAGAC GTTGGTAACA     540
GTGTGTGAGT CTGGAGTTCA GAGTAAGAAC ATCTACCAGA ATCTCTCTCA GTTTTTGCTG     600
CATTACTTAC AGGTCAACTC TACCATATCA GTCAGGGTGG ATAATTTCCA GTATGTGGAT     660
ACAAACACTT TCCCTCTTGA TAATGTGCTA TCCGTCTTCT TATATAGTAG CTCAGACTGA     720
```

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 930 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

```
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TCGTGCCCCT CGGATGCGGC GCTCCTCAGA GATACCGGGC TCCTCGCGGA CGCTGCGCTC     120
CTCTCAGATA CTGTGCGCCC CACAAATGCC GCGCTCCCCA CGGATGCTGC CTACCCTGCG     180
GTTAATGTTC GGGATCGCGA GGCCGCGTGG CCGCCTGCAC TGAACTTCTG TTCCCGCCAC     240
CCAAAGCTCT ATGGCCTAGT CGCTTTGGTT TTGCTGCTTC TGATCGCCGC CTGTGTTCCT     300
ATCTTCACCC GCACCGAGCC TCGGCCAGCG CTCACAATCA CCACCTCGCC CAACCTGGGT     360
ACCCGAGAGA ATAATGCAGA CCAGGTCACC CCTGTTTCCC ACATTGGCTG CCCCAACACT     420
ACACAACAGG GCTCTCCTGT GTTCGCCAAG CTACTGGCTA AAAACCAAGC ATCGTTGTGC     480
AATACAACTC TGAACTGGCA CAGCCAAGAT GGAGCTGGGA GCTCATACCT ATCTCAAGGT     540
CTGAGGTACG AAGAAGACAA AAAGGAGTTG GTGGTAGACA GTCCCGGGCT CTACTACGTA     600
TTTTTGGAAC TGAAGCTCAG TCCAACATTC ACAAACACAG GCCACAAGGT GCAGGGCTGG     660
GTCTCTCTTG TTTTGCAAGC AAAGCCTCAG GTAGATGACT TTGACAACTT GGCCCTGACA     720
GTGGAACTGT TCCCTTGCTC CATGGAGAAC AAGTTAGTGG ACCGTTCCTG GAGTCAACTG     780
TTGCTCCTGA AGGCTGGCCA CCGCCTCAGT GTGGGTCTGA GGGCTTATCT GCATGGAGCC     840
CAGGATGCAT ACAGAGACTG GGAGCTGTCT TATCCCAACA CCACCAGCTT TGGACTCTTT     900
CTTGTGAAAC CCGACAACCC ATGGGAATGA                                      930
```

Claims

1. A method of altering the immunoreactivity of human cells, which method comprising introducing a gene encoding an accessory molecule ligand into said cells so that said accessory molecule ligand is expressed on the surface of said cells.

2. The method of claim 1 wherein the accessory molecule to which the accessory molecule ligand specifically binds is also present on the surface of said human cells.

3. The method of claim 1 wherein said human cells are neoplastic human cells.

4. The method of claim 1 wherein said accessory molecule ligand gene is a chimeric gene.

5. The method in claim 1 wherein said accessory molecule ligand gene is present in a vector capable of transducing human cells.

6. The method of claim 1 wherein said accessory molecule ligand gene is present as part of a genetic vector.

7. The method of claim 1 wherein said accessory molecule ligand gene is operatively linked to a promoter region and a polyadenylation signal.

8. The method of claim 1 wherein said gene is a CD40 ligand gene.

9. The method of claim 7 wherein said CD40 ligand gene is a murine CD40 ligand gene.

10. The method of claim 4 wherein said gene is a chimeric gene which comprises at least a portion of a murine CD40 ligand gene.

11. A method of treating a human neoplasia comprising inserting into said human neoplastic cells a gene which encodes an accessory molecule ligand into said human neoplastic cells so that said accessory molecule ligand is expressed on the surface of said neoplastic cells.

12. The method of claim 11 further comprising:

a) obtaining said human neoplastic cells from a human patient;

b) infusing said human neoplastic cells after having inserted said accessory molecule ligand on the surface of said cells back into said patient.

13. The method of claim 11 wherein the accessory molecule to which the said accessory molecule ligand specifically binds is present on the surface of said human neoplastic cells.

14. The method of claim 11 wherein said accessory molecule ligand gene is a chimeric gene.

15. The method of claim 11 wherein said accessory molecule ligand gene is a chimeric gene which contains at least a portion of the murine CD40 ligand gene.

16. The method of claim 11 wherein said accessory molecule ligand gene is present as part of a genetic vector.

17. The method of claim 11 wherein said accessory molecule ligand gene is operatively linked to a promoter region and a 3′ end region.

18. The method of claim 11 wherein said accessory molecule ligand gene is a CD40 ligand gene.

19. The method of claim 11 wherein the said CD40 ligand gene is a murine CD40 ligand gene.

20. The method of claim 11 wherein said accessory molecule ligand gene is a Fas-ligand gene or a CD27 ligand gene.

21. The method of claim 11 wherein said accessory molecule ligand gene is present in a gene therapy vector.

22. A method of treating a neoplasia in a patient comprising injecting into the tumor bed of said patient a gene which encodes an accessory molecule ligand so that said accessory molecule ligand is expressed on the surface of said tumor cells thereby causing said cells to participate in an immune reaction.

23. A gene therapy vector containing an accessory molecule ligand gene.

24. The gene therapy vector of claim 23 wherein said accessory molecule gene is a CD40 ligand gene.

25. The gene therapy vector of claim 23 wherein said CD40 ligand gene is a murine CD40 ligand gene.

26. The gene therapy vector of claim 23 wherein said CD40 ligand gene is a chimeric gene.

27. The gene therapy vector of claim 23 wherein said chimeric gene contains at least a portion of the murine CD40 ligand gene.

28. The gene therapy vector of claim 23 wherein at least a portion of said vector is derived from adenovirus DNA.

29. The gene therapy vector of claim 23 wherein said vector contains at least a promoter region and a 3' end region.

30. The gene therapy vector of claim 23 wherein said promoter region and said 3' end region are not derived from the same species from which the CD40 ligand gene is derived.

31. The gene therapy vector of claim 23 wherein said portion of said vector is derived from viral DNA.

32. The gene therapy vector of claim 23 wherein at least a portion of said vector is derived from a retrovirus.

33. The gene therapy vector of claim 23 wherein said vector is capable of transducing human cells.

34. The gene therapy vector of claim 23 wherein said vector is capable of transducing animal cells.

35. The gene therapy vector of claim 23 wherein said human cells are human neoplastic cells.

36. The gene therapy vector of claim 23 wherein said human cells are human antigen presenting cells.

37. A genetic construct containing a promoter operatively linked to an accessory molecule ligand gene which is also operatively linked to a polyadenylation signal.

38. A genetic construct in which a promoter is operatively linked to a chimeric accessory molecule ligand gene and a polyadenylation signal.

39. A gene therapy vector containing a chimeric accessory molecule ligand gene.

40. The gene therapy vector of claim 39 wherein said chimeric accessory molecule ligand gene contains at least one gene segment derived from a murine CD40 ligand gene and other gene segments derived from other accessory molecule genes.

41. The gene therapy vector of claim 39 wherein said other accessory molecule ligand genes are human accessory molecule ligand genes.

42. The gene therapy vector of claim 39 wherein said human accessory molecule ligand genes are human CD40 ligand genes.

43. A human cell containing the gene therapy vector of claims 23-36 or 39-42 or the genetic construct of claims 37-38.

44. The human cell of claim 43 wherein said cell is an antigen presenting cell.

45. The human cell of claim 43 wherein said human cell is a neoplastic cell.

46. The human cell of claim 43 wherein said cell is an accessory cell.

47. An animal cell containing the gene therapy vector or genetic construct of claims 23-43.

48. An insect cell containing the gene therapy vector or genetic construct of claims 23-43.

49. A bacterial cell containing the gene therapy vector or genetic construct of claims 23-43.

50. A method of vaccinating an animal against a predetermined organism comprising: administering into an animal to be immunized against a predetermined organism, a vaccine comprising immunogenic antigens capable of causing an immune response to said predetermined organism together with a vector containing a gene including an accessory molecule ligand.

51. The method of claim 50 wherein said immunogenic antigens are encoded by genes present on a genetic vector.

52. The method of claim 50 wherein said gene is a chimeric gene.

53. The method of claim 50 wherein said chimeric gene contains at least a portion of a murine CD40 ligand gene.

54. The method of claim 50 wherein said chimeric gene contains at least a segment of a murine CD40 ligand gene and at least a segment of a different accessory molecule gene.

55. The method of claim 50 wherein said predetermined organism is a virus, a bacteria, a fungus or a neoplastic cell.

56. A method of producing an immune response directed to a predetermined antigen comprising: administering to said animal a genetic vector containing

a gene encoding the antigen to which said immune response is desired together with a genetic vector containing a gene encoding an accessory molecule ligand gene.

57. A chimeric accessory molecule ligand gene comprising at least one domain or sub-domain gene segment derived from a first accessory molecule ligand gene operatively linked to the domain or sub-domain gene segment of a second accessory molecule ligand gene.

58. The chimeric accessory molecule ligand gene of claim 57 in which said first and second accessory molecule ligand genes are selected from the group consisting of the genes from any species encoding a member of the tumor necrosis family, CD40-ligand, Fas-ligand, CD70, TNF_{α}, TNF_{β}, CD30 ligand, 4-1BB ligand (4-1BBL), TNF-related apoptosis inducing ligand (TRAIL) and nerve growth factor.

59. The chimeric accessory molecule ligand gene of claim 57 in which at least one of said domain or sub-domain gene segments is an artificial gene segment.

60. The chimeric accessory molecule encoded by the genes of claims 57-59.

61. A chimeric accessory molecule ligand gene comprising at least a portion of the gene encoding Domains I and II derived from an accessory molecule ligand operatively linked to at least a portion of the gene encoding a Domain of an accessory molecule ligand which in turn is operatively linked to at least a portion of the gene encoding Domain IV of an accessory molecule ligand.

62. The chimeric accessory molecule ligand gene of claim 61 wherein said Domains I and II are derived from the human CD40 ligand gene.

63. The chimeric accessory molecule ligand gene of claim 61 wherein said Domain IV is the human Fas-ligand Domain IV.

64. The chimeric accessory molecule ligand gene of claim 61 wherein said Domain is Domain III of another accessory molecule ligand.

65. The chimeric accessory molecule ligand gene of claim 61 wherein said Domain is a domain from the same accessory molecule ligand.

66. The chimeric accessory molecule ligand gene of claim 61 wherein said Domain is an artificial domain.

67. The method of claim 4 wherein said chimeric gene is a gene of claims 57-60.

68. The method of claim 11 wherein said accessory molecule ligand gene is a chimeric accessory molecule ligand gene of claims 57-60.

69. The gene therapy vector of claim 23 wherein said chimeric gene is a chimeric accessory molecule ligand gene of claims 57-60.

70. A method of treating rheumatoid arthritis in a joint comprising inserting into the joint a vector containing a gene which encodes an accessory molecule ligand so that said accessory molecule ligand is expressed on the surface of cells within the joint.

71. The method of claim 70 wherein said accessory molecule ligand gene is a chimeric accessory molecule ligand gene which is comprised of at least a portion of a human Fas-ligand gene.

72. The method of claim 70 wherein said accessory molecule ligand gene is a chimeric accessory molecule ligand gene which contains at least a portion of the murine Fas-ligand gene.

73. The method of claim 70 wherein said accessory molecule ligand gene is a murine Fas-ligand gene.

74. The method of claim 70 wherein said accessory molecule ligand gene is the murine Fas-ligand gene.

75. The method of claim 70 wherein said accessory molecule ligand gene is a chimeric accessory molecule gene comprised of at least a portion of domain III from the murine Fas-ligand gene and a portion of domain IV from the human Fas-ligand gene.

76. The method of claim 70 wherein said accessory molecule ligand gene is a chimeric accessory molecule ligand gene comprised of a portion of domain III of the human CD70 gene and at least a portion of domain IV of the human Fas-ligand gene.

77. A method of treating rheumatoid arthritis in a joint comprising inserting into the joint cells which have been transformed with a gene which encodes on accessory molecule ligand which is expressed on the surface of said cells.

78. A chimeric accessory molecule ligand comprised of at least a portion of the fourth domain of human Fas-ligand.

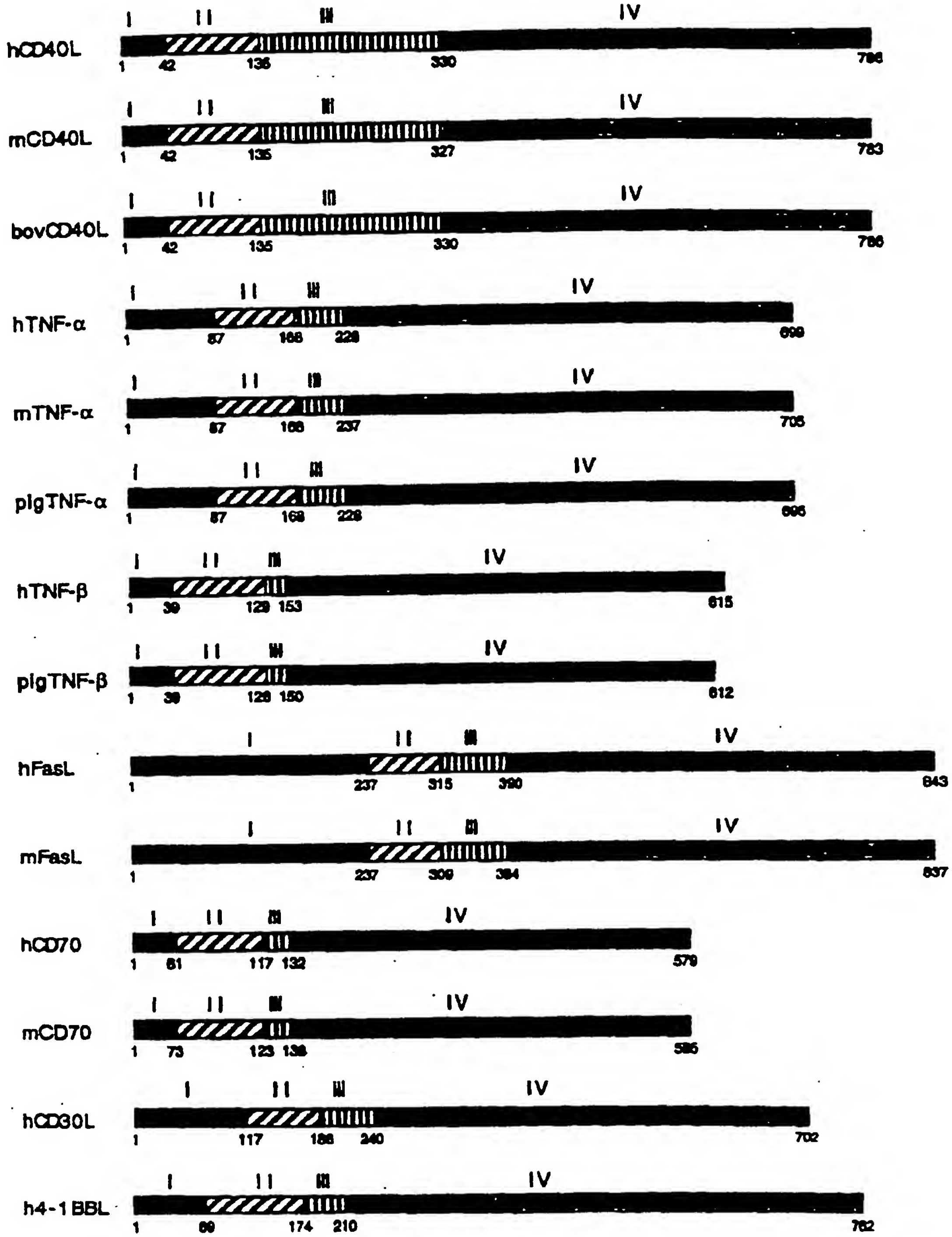
79. A chimeric accessory molecule ligand derived from a Fas-ligand in which at least one matrix metalloproteinase cleavage site has been removed.

80. A chimeric accessory molecule ligand comprised of domain III of the Murine Fas-ligand or the human CD70 gene, and domain IV of the human Fas-ligand.

81. A gene therapy vector containing a gene encoding chimeric accessory molecule of claims 78-80.

82. A cell containing a gene therapy vector of claim 81.

83. A method of altering the immunoreactivity of human cells, which method comprising introducing a gene encoding an accessory molecule ligand which has a stabilized activity into said cells so that said accessory molecule ligand is expressed on the surface of said cells.



DOMAINS: I - Cytoplasmic Domain; II - Transmembrane Domain; III - Proximal Extracellular Domain; IV - Distal Extracellular Domain (putative soluble form)

Figure 1

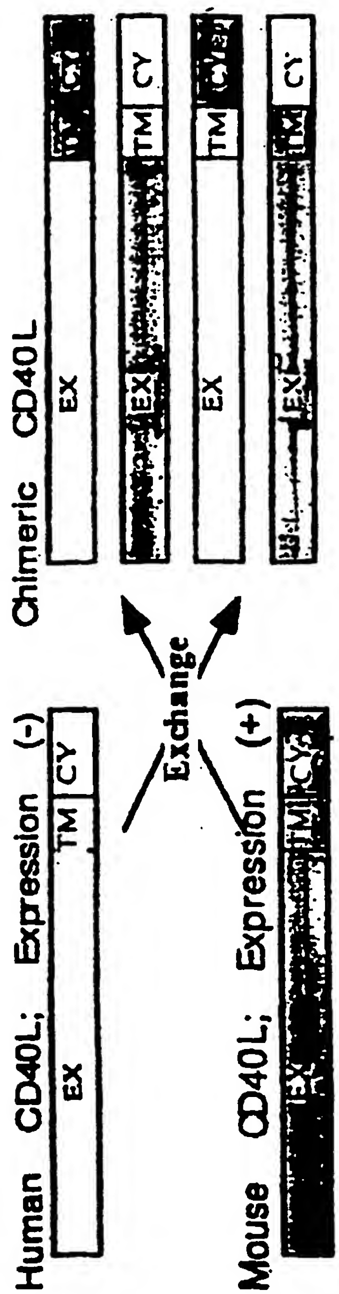

Human CD40L; Expression (-)
EX
TM
CY
Mouse CD40L; Expression (+)
EX
TM
CY
Exchange
Chimeric CD40L
EX
TM
CY

Figure 2

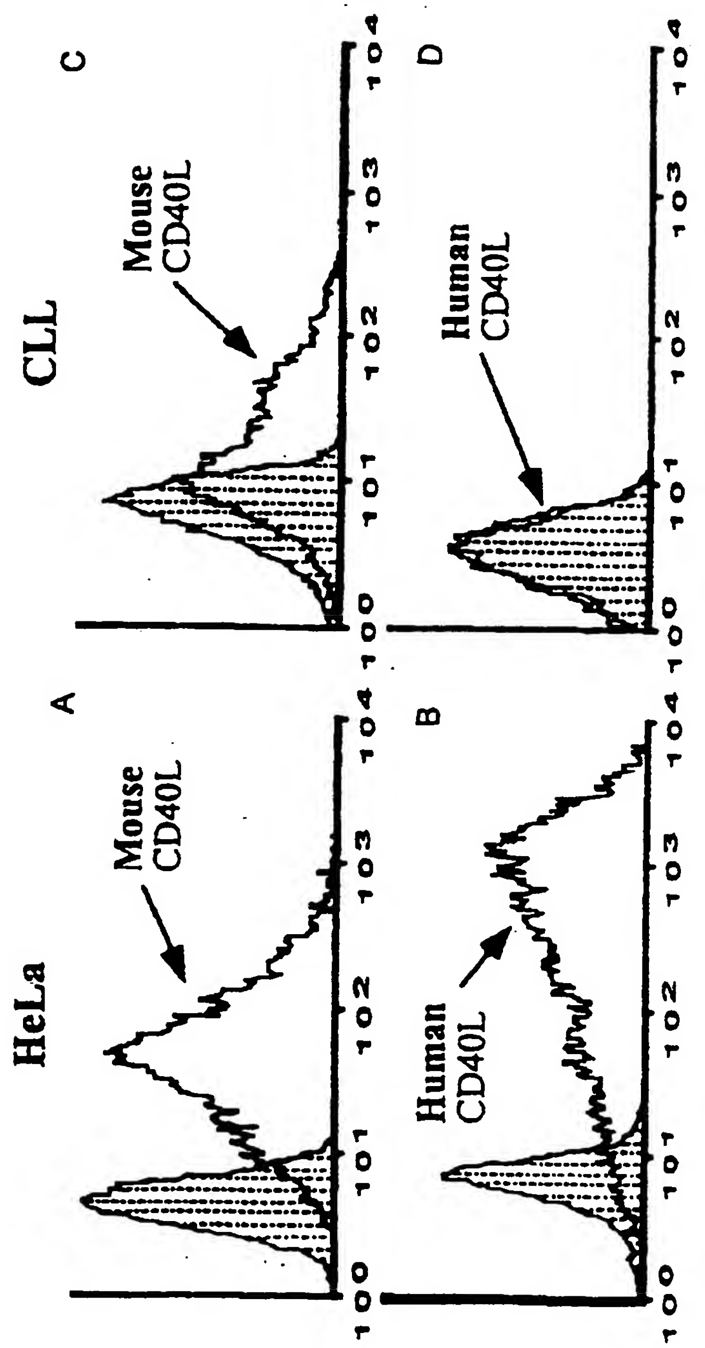

HeLa
CLL
A
B
C
D
Mouse CD40L
Mouse CD40L
Human CD40L
Human CD40L
10^0
10^1
10^2
10^3
10^4

Figure 3

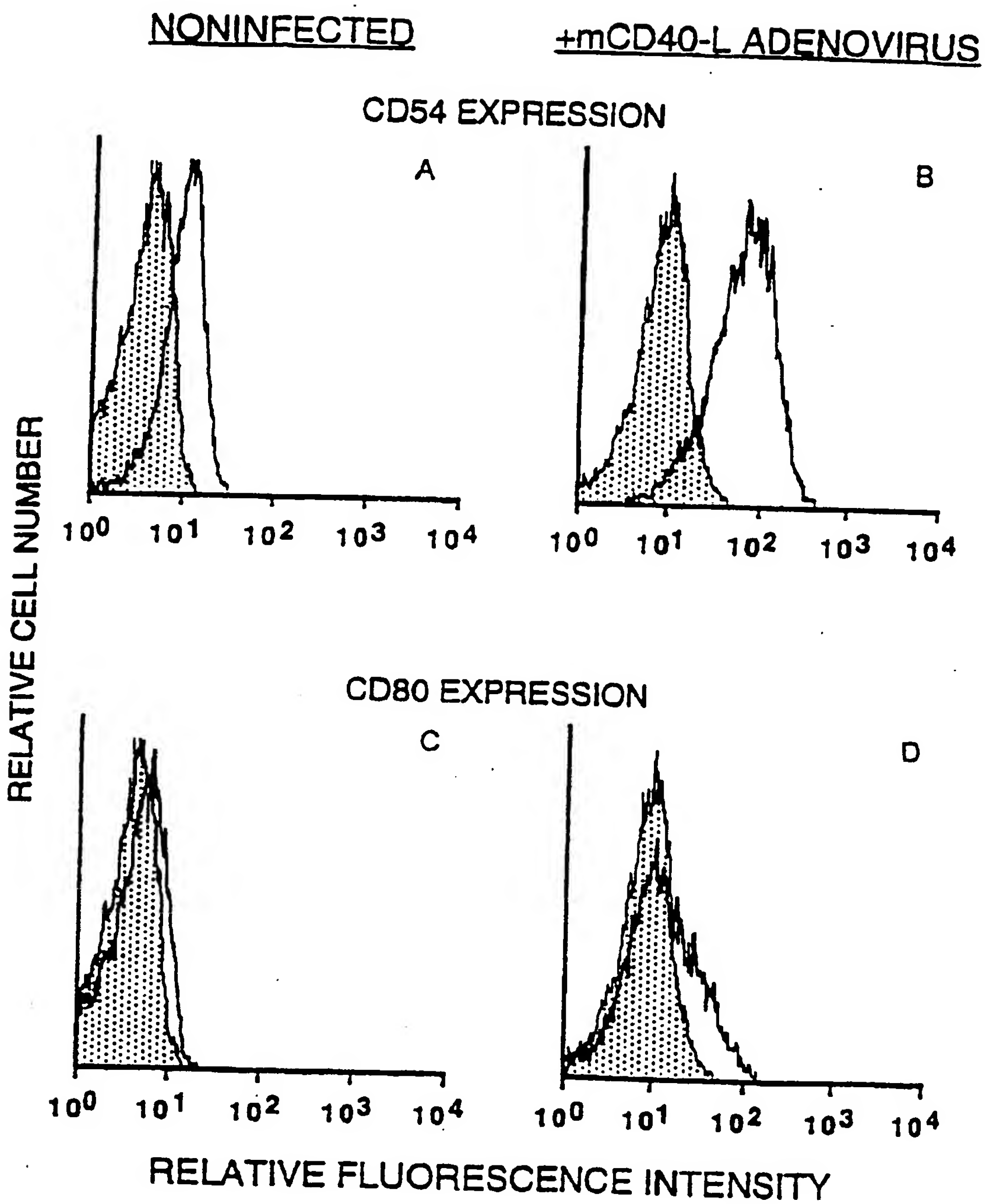

NONINFECTED
+mCD40-L ADENOVIRUS
CD54 EXPRESSION
A
B
RELATIVE CELL NUMBER
10^0 10^1 10^2 10^3 10^4
CD80 EXPRESSION
C
D
RELATIVE FLUORESCENCE INTENSITY

Figure 4

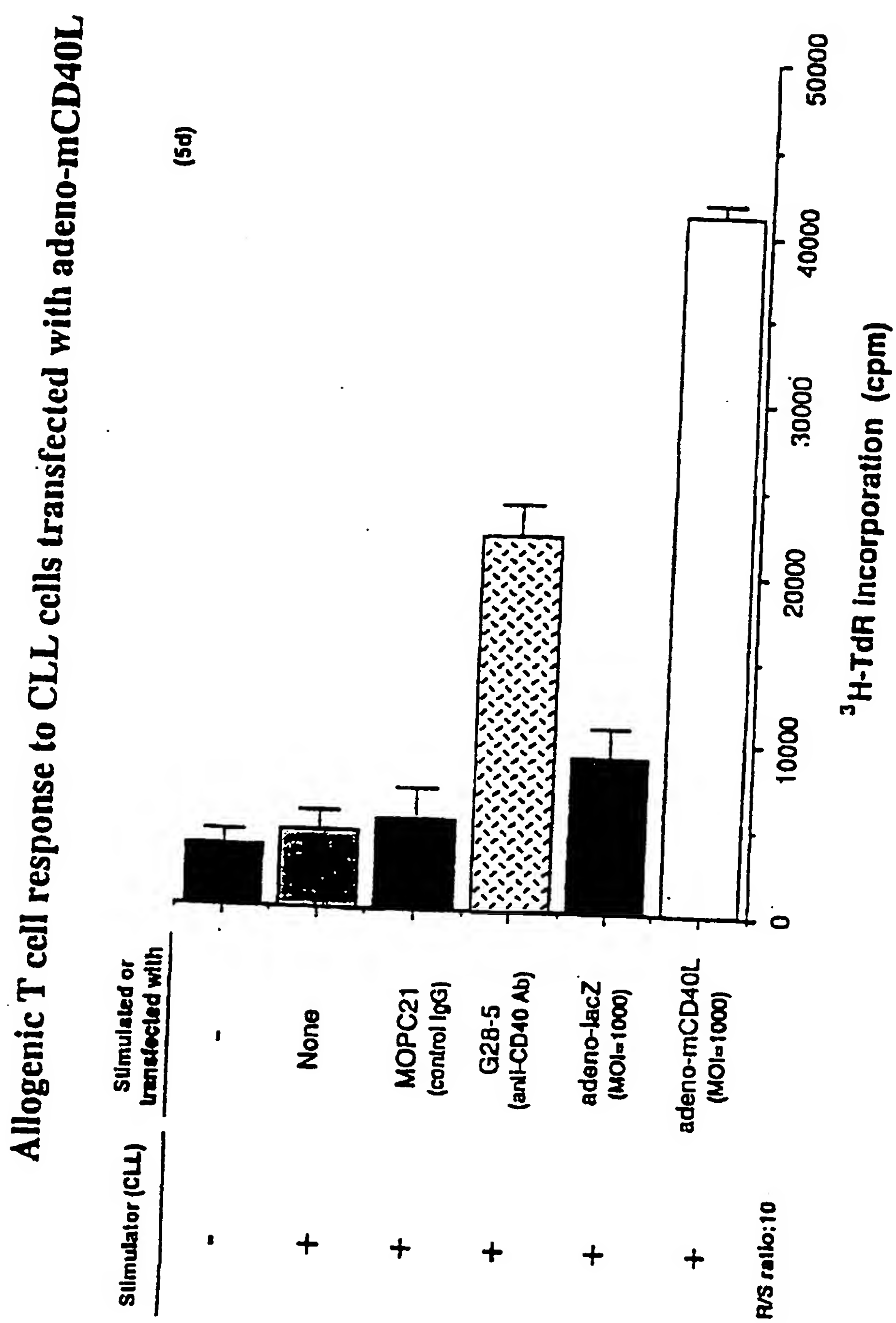

Allogenic T cell response to CLL cells transfected with adeno-mCD40L
(5d)
Stimulator (CLL)
Stimulated or transfected with
-
+
None
+
MOPC21 (control IgG)
+
G28-5 (anti-CD40 Ab)
+
adeno-lacZ (MOI=1000)
+
adeno-mCD40L (MOI=1000)
R/S ratio:10
0
10000
20000
30000
40000
50000
^{3}H-TdR Incorporation (cpm)

Figure 5

Production of IFNγ by allogenic T lymphocytes stimulated with CLL B cells

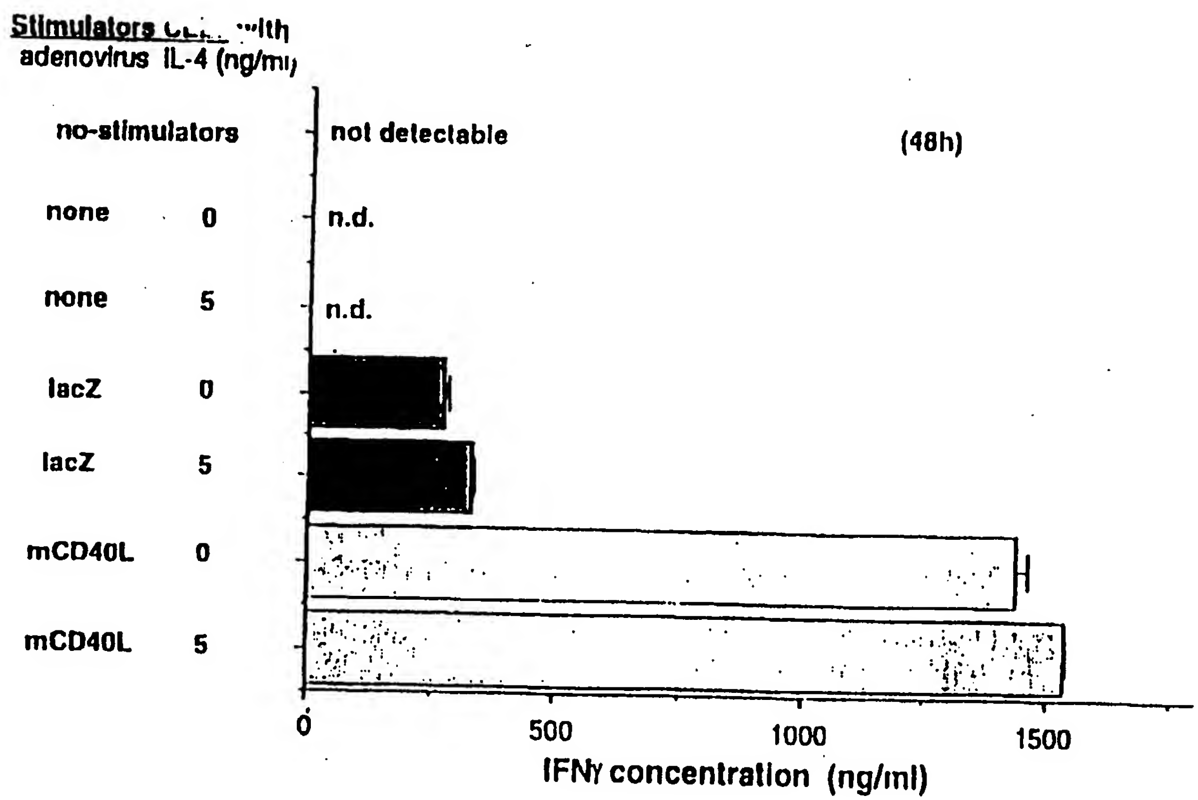


Figure 6

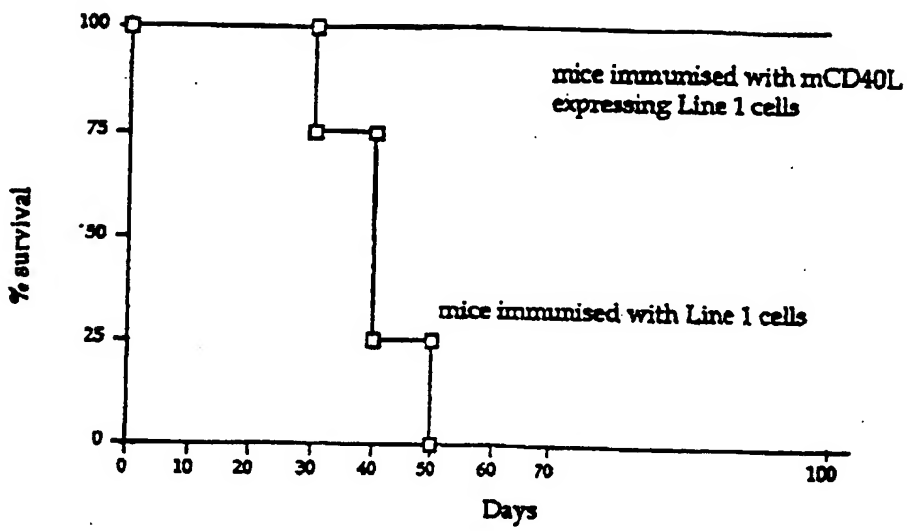

100
75
50
25
0
% survival
0
10
20
30
40
50
60
70
100
Days
mice immunised with mCD40L expressing Line 1 cells
mice immunised with Line 1 cells

Figure 7

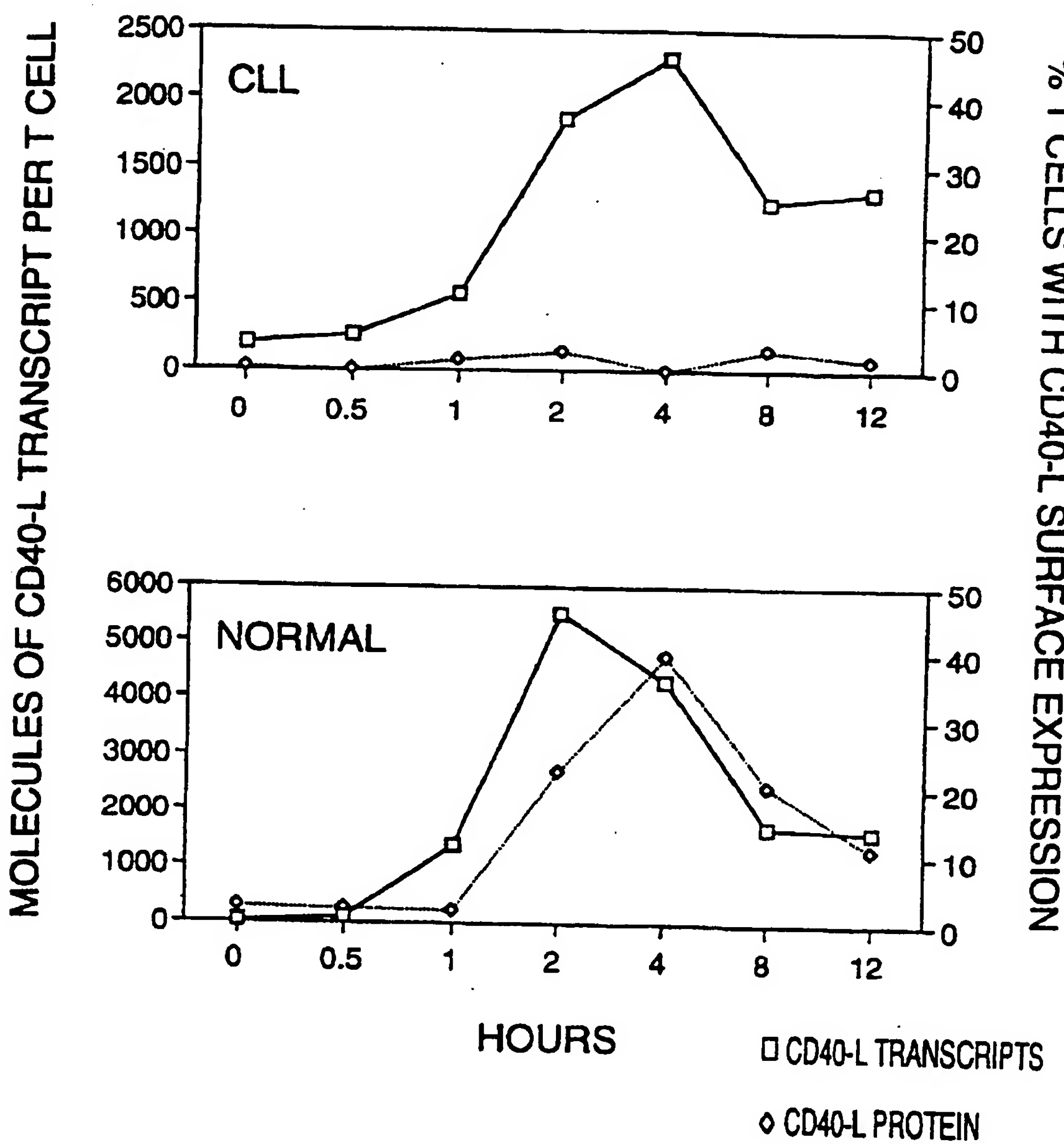

MOLECULES OF CD40-L TRANSCRIPT PER T CELL
% T CELLS WITH CD40-L SURFACE EXPRESSION
CLL
2500
2000
1500
1000
500
0
50
40
30
20
10
0
0
0.5
1
2
4
8
12
NORMAL
6000
5000
4000
3000
2000
1000
0
50
40
30
20
10
0
0
0.5
1
2
4
8
12
HOURS
CD40-L TRANSCRIPTS
CD40-L PROTEIN

Figure 8

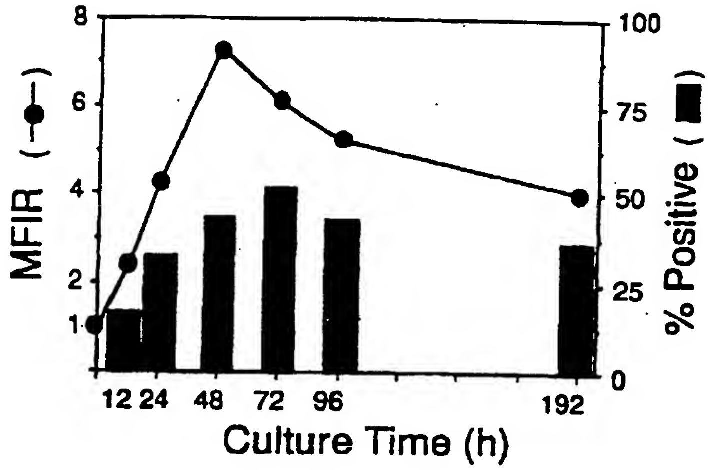

MFIR (—●—)
% Positive (■)
8
6
4
2
1
100
75
50
25
0
12 24 48 72 96 192
Culture Time (h)

Figure 9

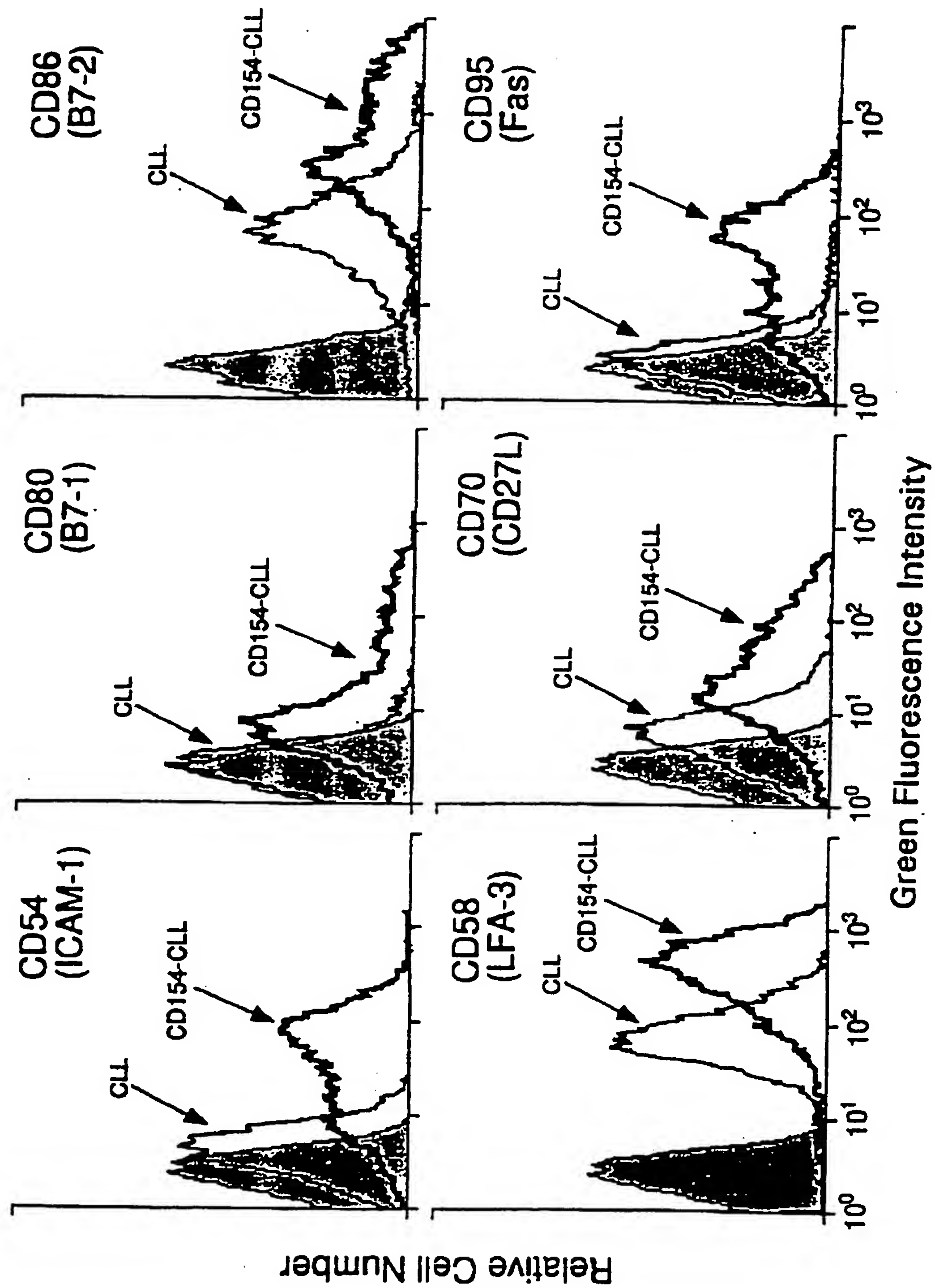

CD54
(ICAM-1)
CD80
(B7-1)
CD86
(B7-2)
CD58
(LFA-3)
CD70
(CD27L)
CD95
(Fas)
CLL
CD154-CLL
Relative Cell Number
Green Fluorescence Intensity
10^0
10^1
10^2
10^3

Figure 10

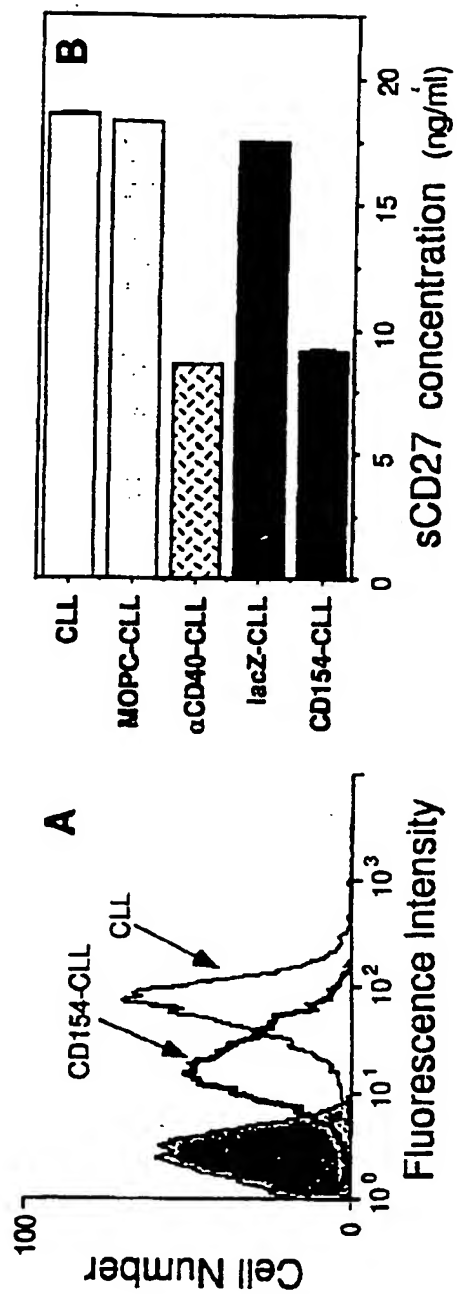

A
Cell Number
100
0
CD154-CLL
CLL
10^0
10^1
10^2
10^3
Fluorescence Intensity
B
CLL
MOPC-CLL
αCD40-CLL
lacZ-CLL
CD154-CLL
0
5
10
15
20
sCD27 concentration (ng/ml)

Figure 11

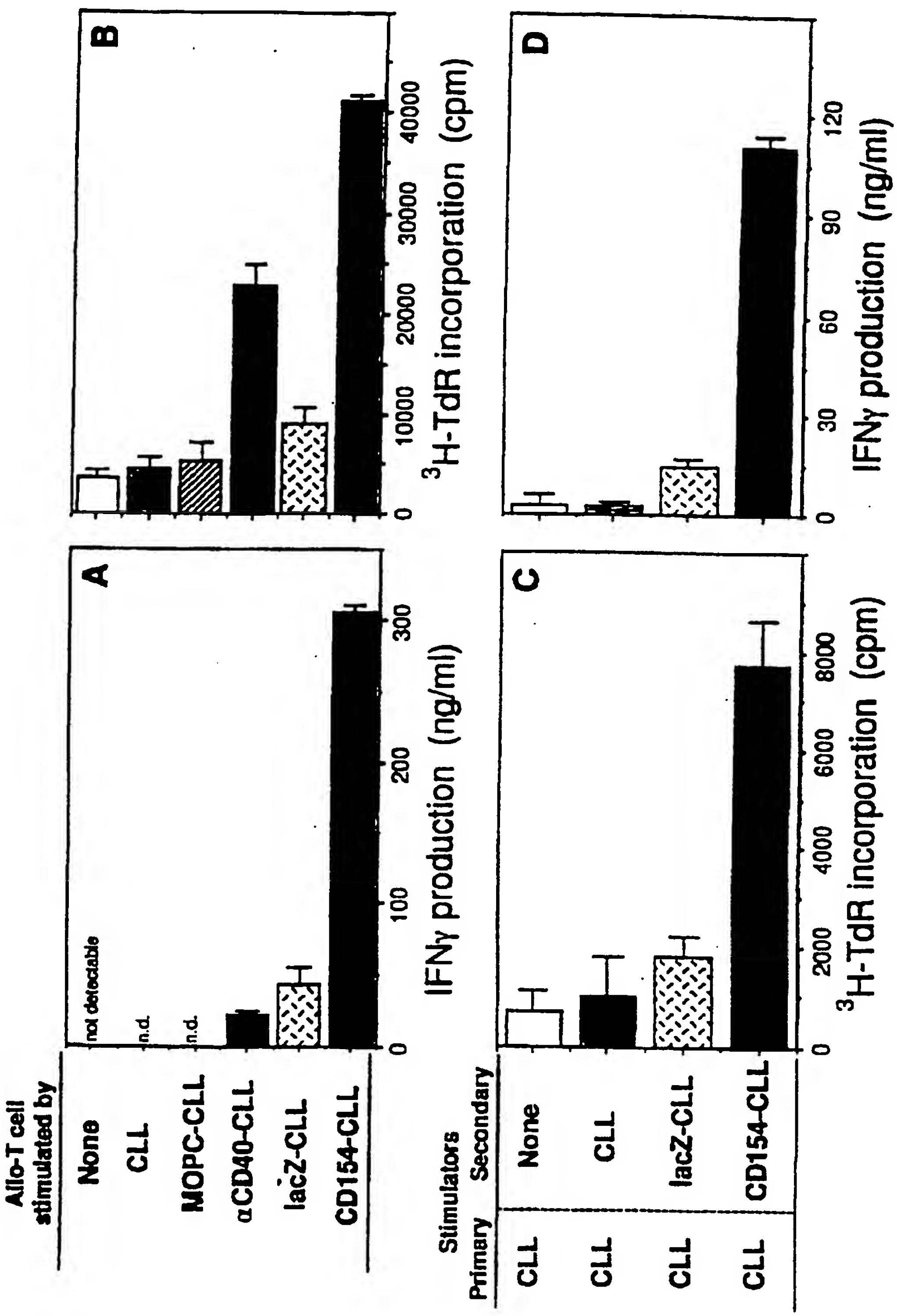

A
B
C
D
Allo-T cell stimulated by
None
CLL
MOPC-CLL
αCD40-CLL
lacZ-CLL
CD154-CLL
not detectable
n.d.
n.d.
IFNγ production (ng/ml)
0
100
200
300
^{3}H-TdR incorporation (cpm)
0
10000
20000
30000
40000
0
30
60
90
120
0
2000
4000
6000
8000
Stimulators
Primary
Secondary
CLL
None
CLL
CLL
CLL
lacZ-CLL
CLL
CD154-CLL

Figure 12

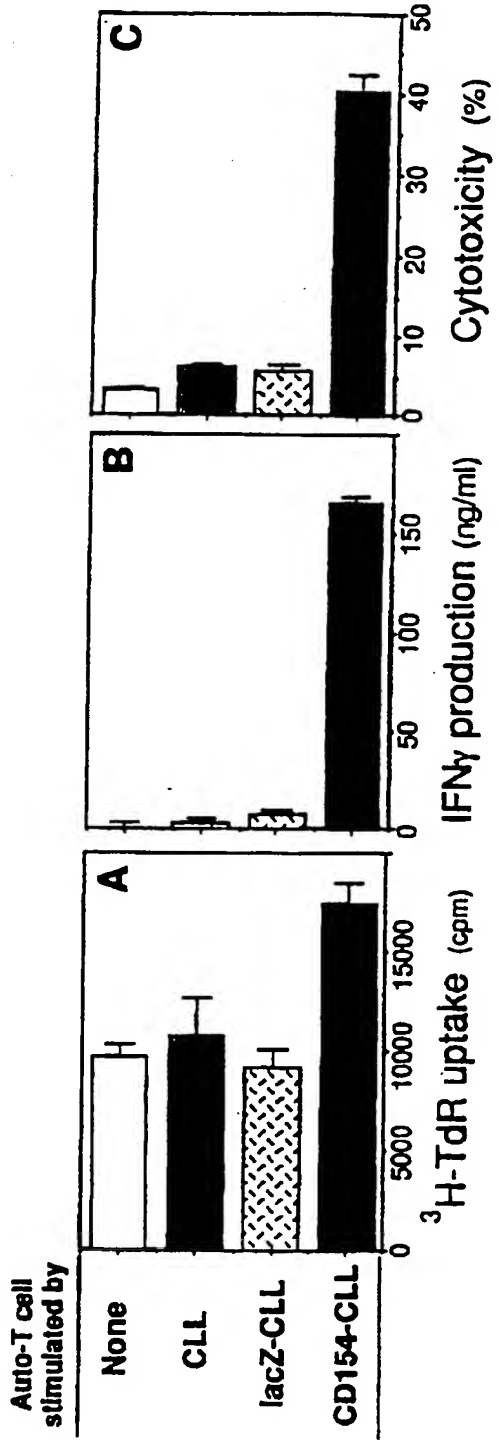

Auto-T cell stimulated by
None
CLL
lacZ-CLL
CD154-CLL
A
B
C
0
5000
10000
15000
^{3}H-TdR uptake (cpm)
0
50
100
150
IFNγ production (ng/ml)
0
10
20
30
40
50
Cytotoxicity (%)

Figure 13

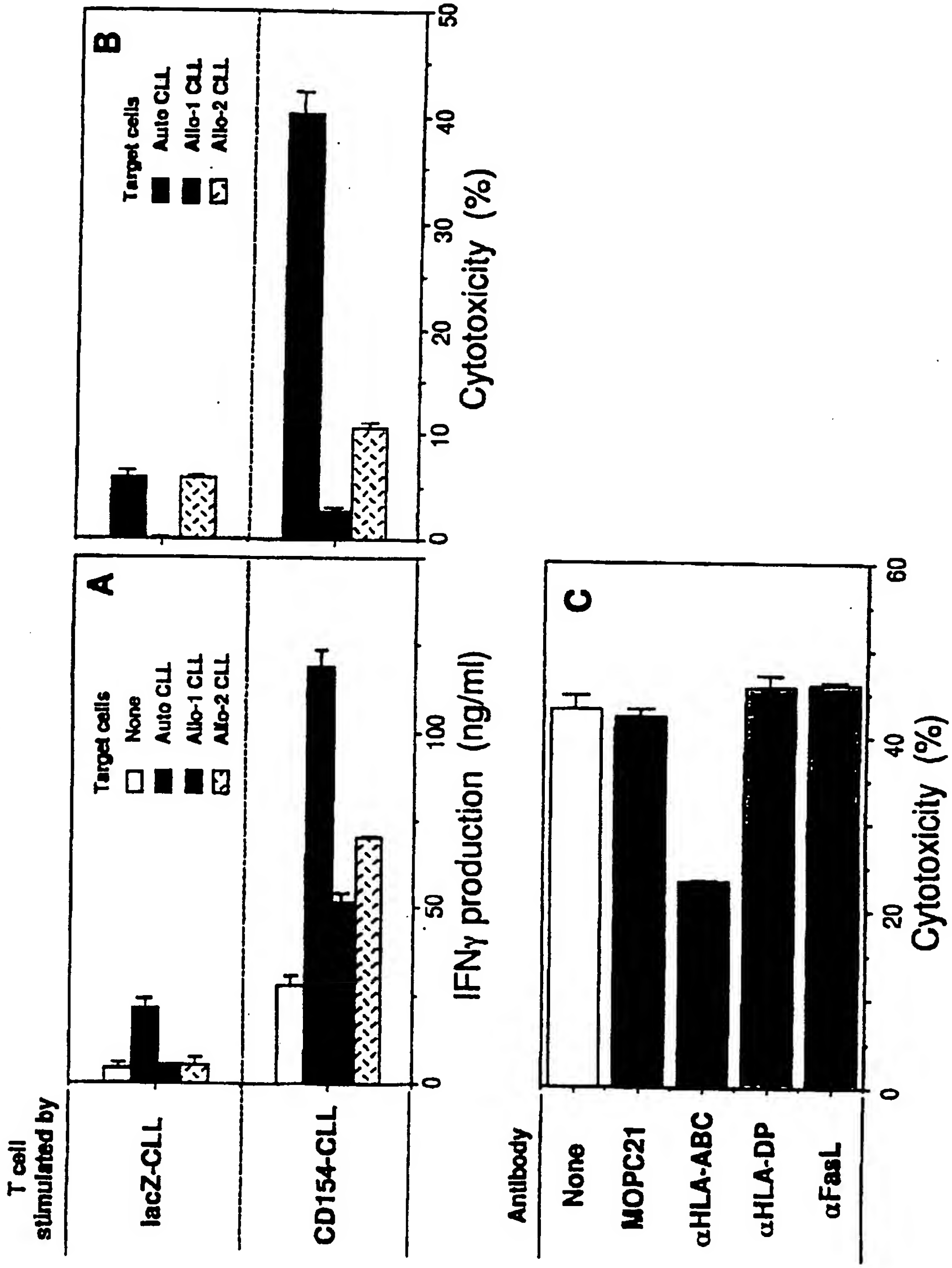

A
B
C
Target cells
None
Auto CLL
Allo-1 CLL
Allo-2 CLL
T cell stimulated by
lacZ-CLL
CD154-CLL
IFNγ production (ng/ml)
0
50
100
Cytotoxicity (%)
0
10
20
30
40
50
Antibody
None
MOPC21
αHLA-ABC
αHLA-DP
αFasL
0
20
40
60

Figure 14

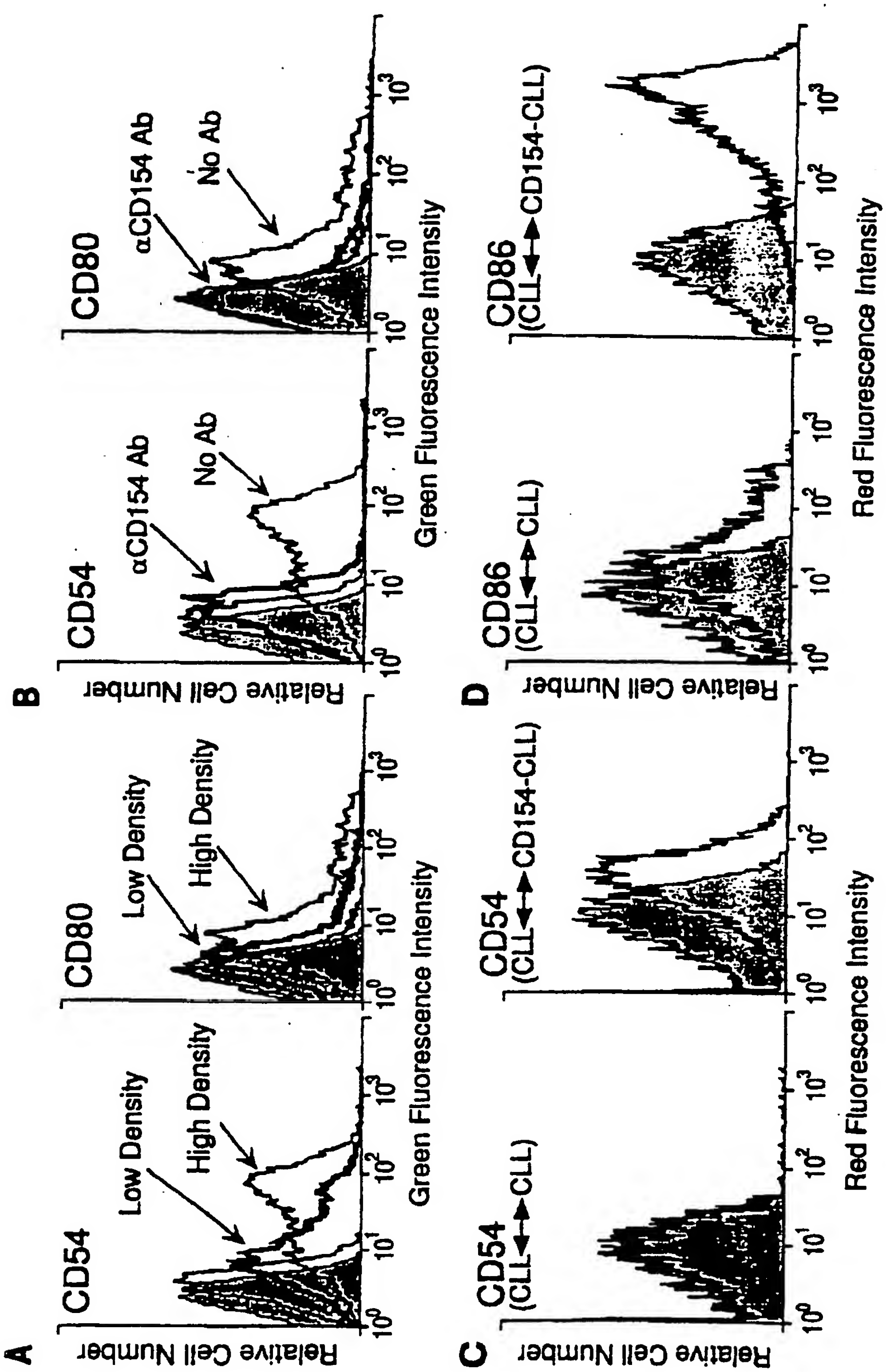

A
CD54
Low Density
High Density
CD80
Low Density
High Density
Relative Cell Number
Green Fluorescence Intensity
B
CD54
αCD154 Ab
No Ab
CD80
αCD154 Ab
No Ab
Relative Cell Number
Green Fluorescence Intensity
C
CD54 (CLL↔CLL)
CD54 (CLL↔CD154-CLL)
Relative Cell Number
Red Fluorescence Intensity
D
CD86 (CLL↔CLL)
CD86 (CLL↔CD154-CLL)
Relative Cell Number
Red Fluorescence Intensity

Figure 15

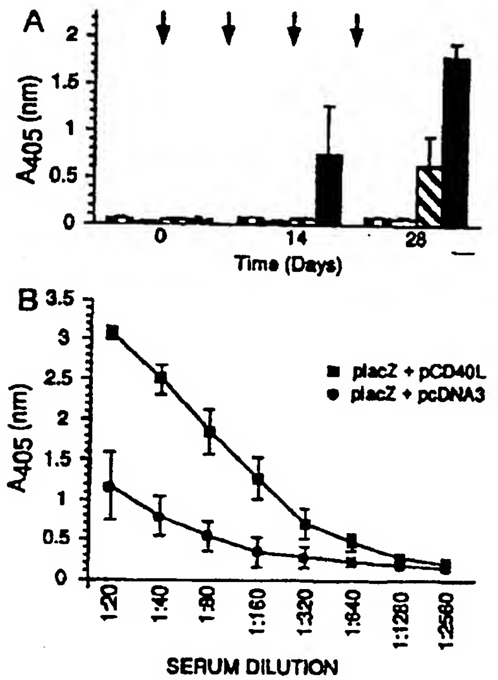

A
A405 (nm)
2
1.5
1
0.5
0
0
14
28
Time (Days)
B
3.5
3
2.5
2
1.5
1
0.5
0
A405 (nm)
placZ + pCD40L
placZ + pcDNA3
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
1:2560
SERUM DILUTION

Figure 16

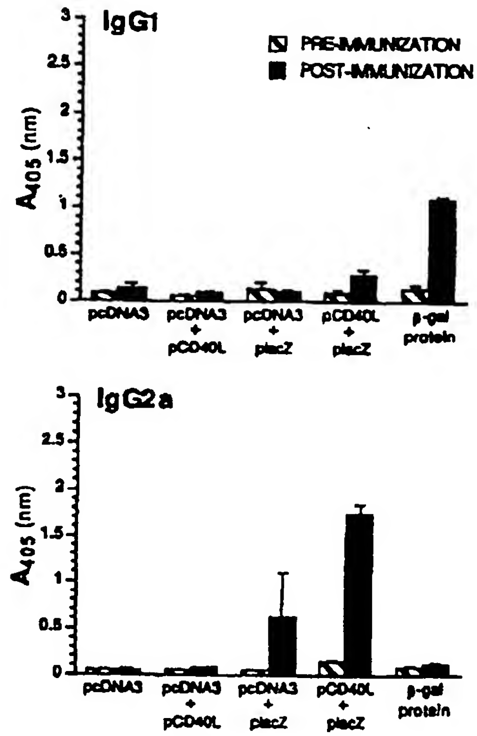

IgG1
PRE-IMMUNIZATION
POST-IMMUNIZATION
A_{405} (nm)
3
2.5
2
1.5
1
0.5
0
pcDNA3
pcDNA3 + pCD40L
pcDNA3 + placZ
pCD40L + placZ
β-gal protein
IgG2a
A_{405} (nm)
3
2.5
2
1.5
1
0.5
0
pcDNA3
pcDNA3 + pCD40L
pcDNA3 + placZ
pCD40L + placZ
β-gal protein

Figure 17

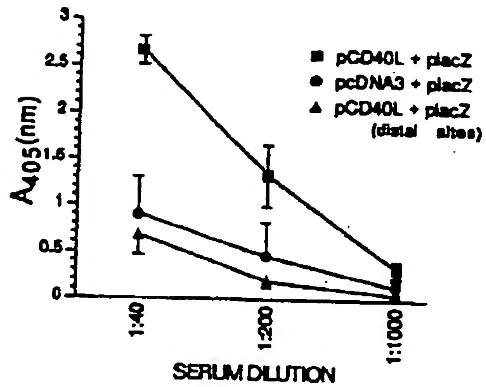

3
2.5
2
1.5
1
0.5
0
A_{405}(nm)
pCD40L + placZ
pcDNA3 + placZ
pCD40L + placZ
(distal sites)
1:40
1:200
1:1000
SERUM DILUTION

Figure 18

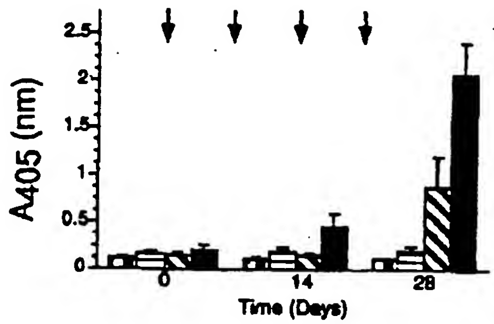


Figure 19

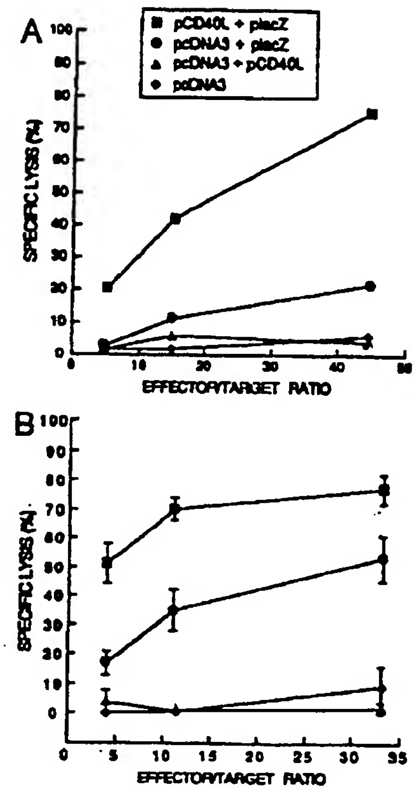

A
pCD40L + placZ
pcDNA3 + placZ
pcDNA3 + pCD40L
pcDNA3
SPECIFIC LYSIS (%)
EFFECTOR/TARGET RATIO
B
SPECIFIC LYSIS (%)
EFFECTOR/TARGET RATIO

Figure 20

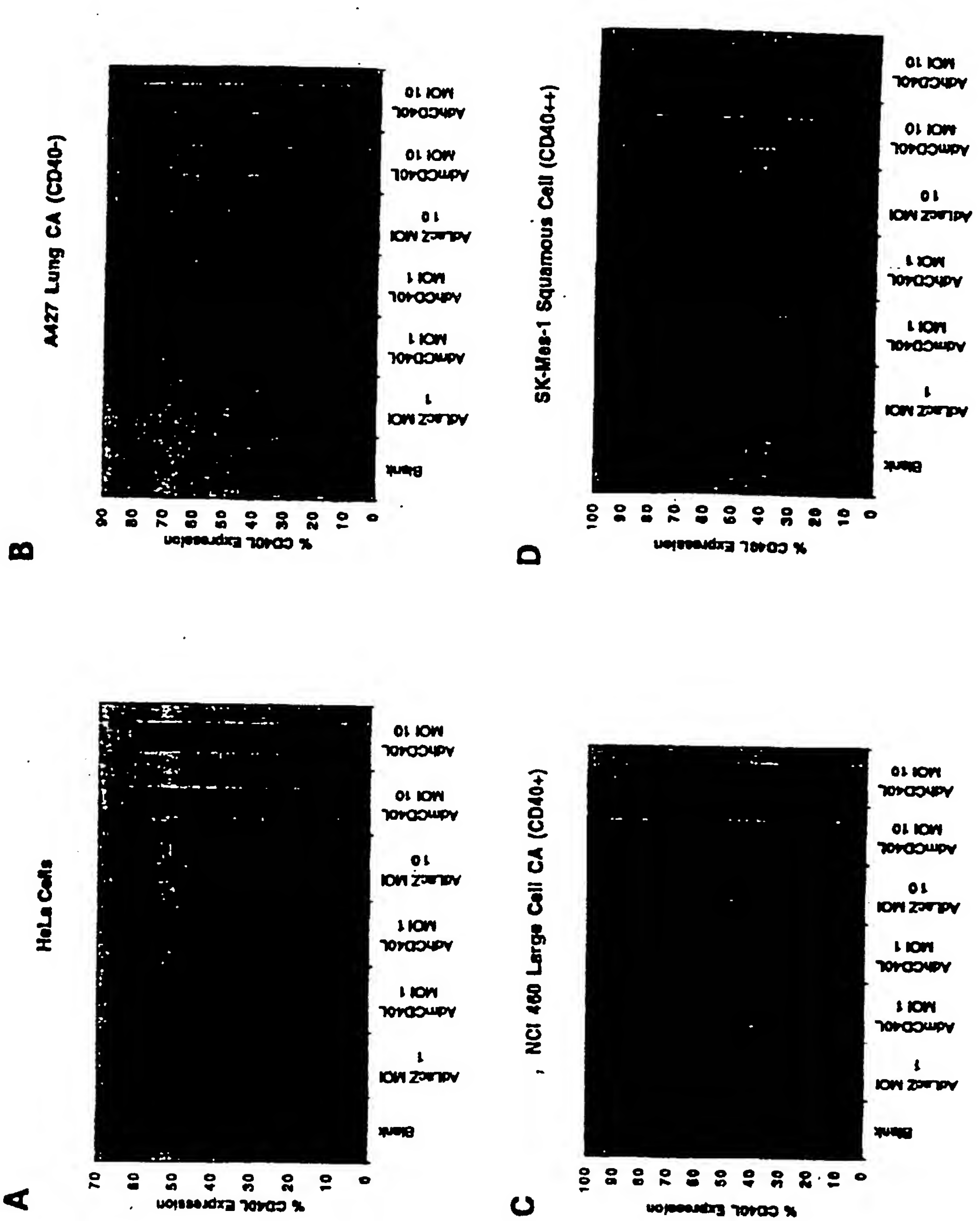
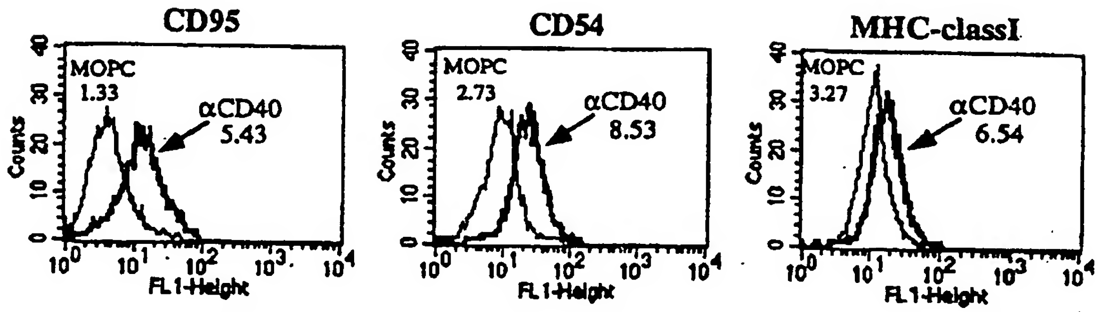

A
HeLa Cells
B
A427 Lung CA (CD40-)
C
NCI 460 Large Cell CA (CD40+)
D
SK-Mes-1 Squamous Cell (CD40++)
% CD40L Expression
Blank
AdLacZ MOI 1
AdmCD40L MOI 1
AdhCD40L MOI 1
AdLacZ MOI 10
AdmCD40L MOI 10
AdhCD40L MOI 10

Figure 21

A


CD95
MOPC
1.33
αCD40
5.43
CD54
MOPC
2.73
αCD40
8.53
MHC-classI
MOPC
3.27
αCD40
6.54
Counts
FL1-Height

B

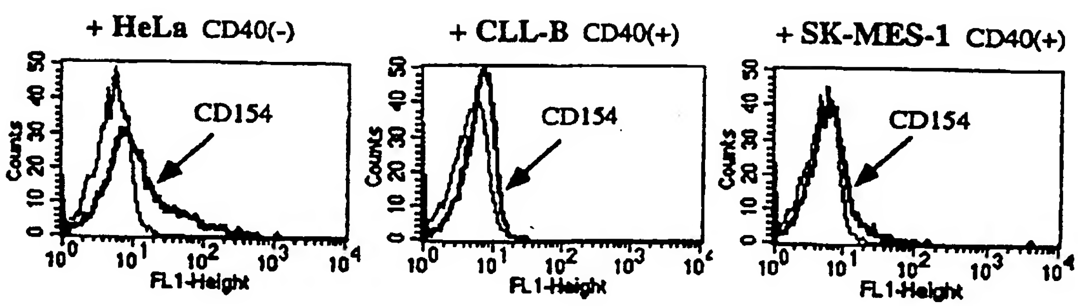

+ HeLa CD40(-)
CD154
+ CLL-B CD40(+)
CD154
+ SK-MES-1 CD40(+)
CD154
Counts
FL1-Height

Figure 22

RA SYNOVIAL FLUID AND PLASMA INHIBITION OF FAS-LIGAND

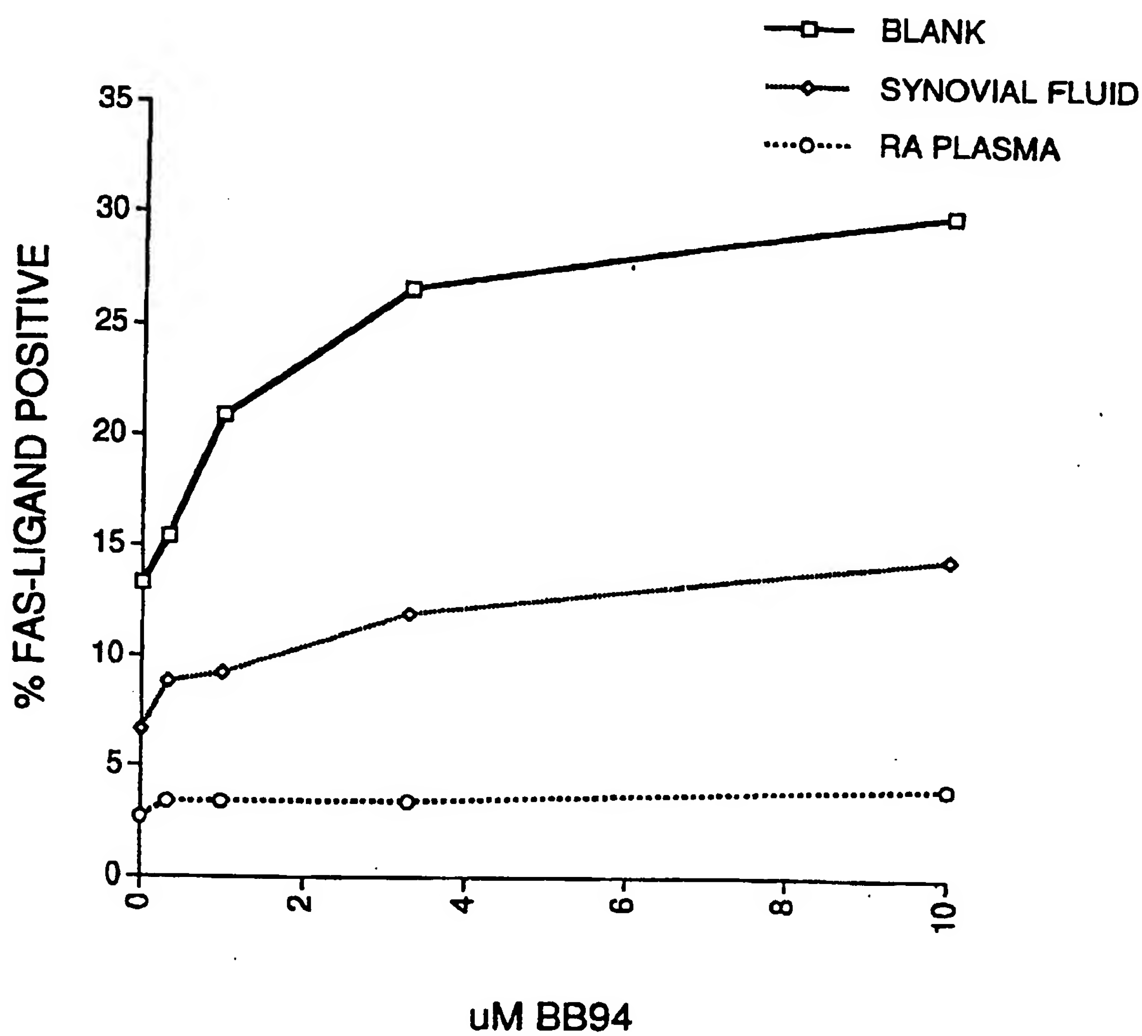


Figure 23

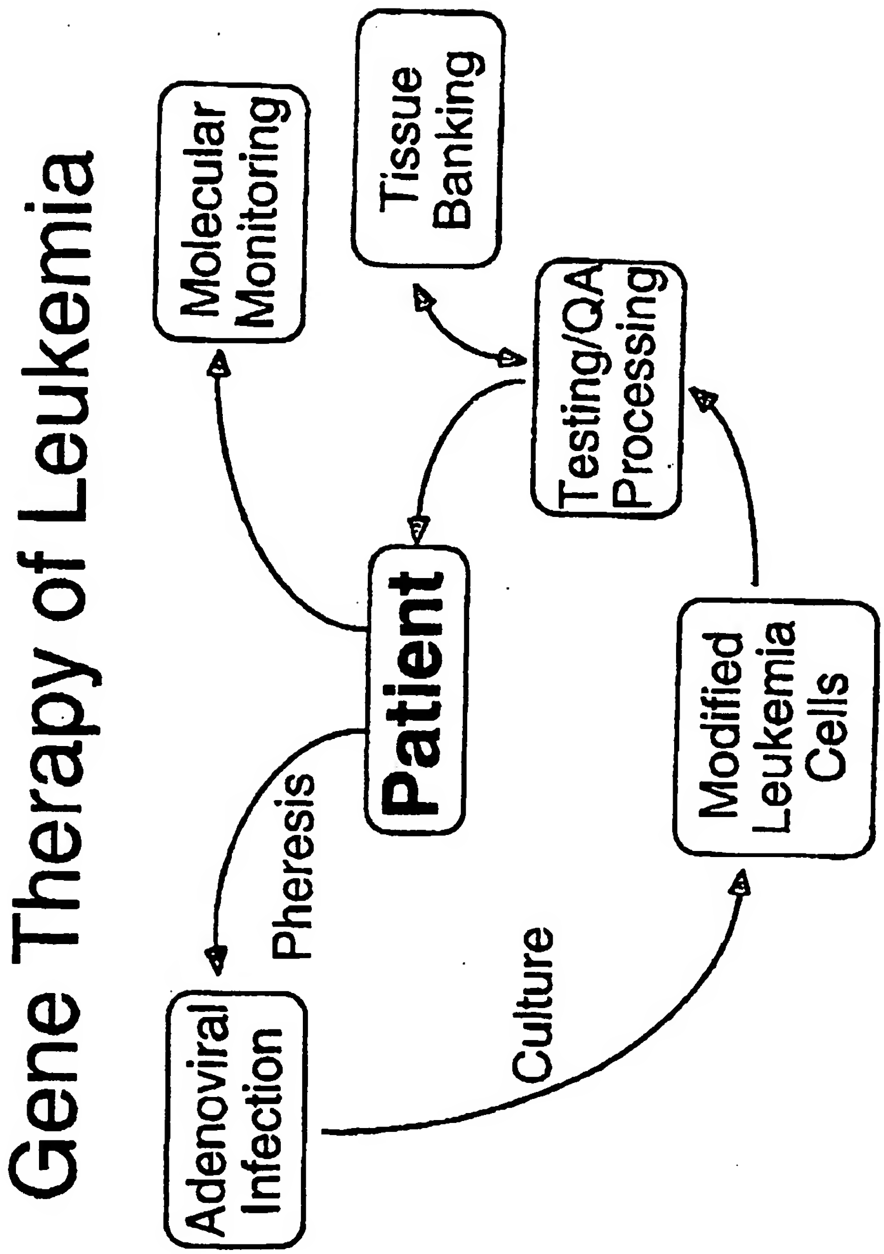

Gene Therapy of Leukemia
Patient
Pheresis
Adenoviral Infection
Culture
Modified Leukemia Cells
Testing/QA Processing
Tissue Banking
Molecular Monitoring

Figure 24

```
  1 MQQPFNYPYPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPPP  50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 MQQPFNYPYPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPPP  50

 51 PPPLPPPPPPPPLPPLPLPPLKKRGNHSTGLCLLVMFFMVLVALVGLGLG 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 PPPLPPPPPPPPLPPLPLPPLKKRGNHSTGLCLLVMFFMVLVALVGLGLG 100

101 MFQLFHLQKELAELREETSQMHTASSLEKQIGHPSPPPEKKELRKVAHLT 150
    |||||||||||||||| |.|  ..||:|||||||||||||||||||||||
101 MFQLFHLQKELAELREFTNQSLKVSSFEKQIGHPSPPPEKKELRKVAHLT 150

151 GKSNSRSMPLEWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYFRGQ 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 GKSNSRSMPLEWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYFRGQ 200

201 SCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWARSSYLGAVFN 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 SCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWARSSYLGAVFN 250

251 LTSADHLYVNVSELSLVNFEESQTFFGLYKL 281
    |||||||||||||||||||||||||||||||
251 LTSADHLYVNVSELSLVNFEESQTFFGLYKL 281
```

Figure 25

```
  1 MQQPFNYPYPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPPP 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 MQQPFNYPYPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPPP 50

 51 PPPLPPPPPPPPLPPLPLPPLKKRGNHSTGLCLLVMFFMVLVALVGLGLG 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 PPPLPPPPPPPPLPPLPLPPLKKRGNHSTGLCLLVMFFMVLVALVGLGLG 100

101 MFQLFHLQKELAELRESTSQMHTASSLEKQIGHPSPPPEKKELRKVAHLT 150
    |||||:                    :...||||||||||||||||||||
101 MFQLFR....................FAQAIGHPSPPPEKKELRKVAHLT 130

151 GKSNSRSMPLEWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYFRGQ 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
131 GKSNSRSMPLEWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYFRGQ 180

201 SCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWARSSYLGAVFN 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
181 SCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWARSSYLGAVFN 230

251 LTSADHLYVNVSELSLVNFEESQTFFGLYKL 281
    |||||||||||||||||||||||||||||||
231 LTSADHLYVNVSELSLVNFEESQTFFGLYKL 261
```

Figure 26

```
                 .         .         .         .
  1 MQQPFNYPYPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPPP 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 MQQPFNYPYPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPPP 50
              .         .         .         .         .
 51 PPPLPPPPPPPPLPPLPLPPLKKRGNHSTGLCLLVMFFMVLVALVGLGLG 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 PPPLPPPPPPPPLPPLPLPPLKKRGNHSTGLCLLVMFFMVLVALVGLGLG 100
              .         .         .         .         .
101 MFQLFHLQKELAELRESTSQMHTASSLEKQIGHPSPPPEKKELRKVAHLT 150
    |||||     ::|  .:.| .: : :   .||||||||||||||||||||
101 MFQLP.....MPKEGSGCSVRRRPYGCVLRIGHPSPPPEKKELRKVAHLT 145
              .         .         .         .         .
151 GKSNSRSMPLEWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYFRGQ 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
146 GKSNSRSMPLSWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYFRGQ 195
              .         .         .         .         .
201 SCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWARSSYLGAVFN 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
196 SCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWARSSYLGAVFN 245
              .         .         .
251 LTSADHLYVNVSELSLVNFEESQTFFGLYKL 281
    |||||||||||||||||||||||||||||||
246 LTSADHLYVNVSELSLVNFEESQTFFGLYKL 276
```

Figure 27

Matrix Metalloproteinase Cleavage Sites

P_4	P_3	Cleavage ↓ P_2	P_1	P'_1	P'_2	P'_3	P'_4

Collagenases

MMP-1 Interstitial Collagenase

P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
Ala	Pro	Leu	Gly	Met	Arg	Met/Ala	Arg
Gly/Leu	Leu	Met/Tyr	His	Leu	Leu	Gly	Lys
Met	Ala	Val/Gly	Glu	Ile	Phe	Val	Gln
Glu	Asp	Ile	Tyr	Gln	Trp	Ser	Ile
Pro	Ser	Gln/Arg	Ala	Pro	Glu	Glu	Gly
Tyr	Glu	Asp	Phe	Phe	Ala	Phe	S r
Ile	Gly	Glu	Gln	Ala	Val/Gly	Arg	Glu
Thr	Arg	Ala	Asn	Tyr/Val	Ser	Pro	Ala
Arg				[not K,E,W]	Asn		

MMP-8 Neutrophil Collagenase

P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
Ala	Pro	Leu	Glu	Tyr	Ala	Gly	Arg
Gly/Leu	Leu	Gln	Gly/His	Ile	Leu	Met	Gln
Met			Ala	Leu	Trp	Ala	
Glu				Val			
Pro				Phe			
Tyr/Ile/Thr/Arg			Ala				

(otherwise same as MMP-1)

Figure 28A

Gelatinases

MMP-2 Gelatinase A

P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
Gly	Pro	Arg	Gly	Leu	Ala/Leu	Gly/Ala	Gln
Ile	Ala	Gln	Asn	Ile/Phe	Phe/Trp	Leu	Arg
Pro	Arg	Leu	Ala	Val/Met	Gly	Ser	His
Arg		Ala	His	Ala	Arg/Gln	Pro	Pro
Leu		Lys	Leu	Glu	His		
		Ile	Tyr	Gln/Asn	Val		
		His		Ser			

MMP-9 Gelatinase B

P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
	Pro	Arg	Gly	Leu	Glu	Ala	Thr
		Gln		Ile/Phe	Ala/Leu/Phe	Leu	
Gln/Arg							
		Leu		Val/Met	Trp/Gly	Ser	
				Ala		Gly	

Stromelysins

MMP-3 Stromelysin 1

P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
Asp	Pro	Phe	Glu	Leu	Arg	Ala	Thr
Gly	Ala	Leu/Met	Ala	Phe	Leu/Phe	Arg/Met	
Gln/Arg							
Leu	Val	Tyr	Gln/Phe	Trp/Tyr	Trp	Gly	Pro
Ile	Leu	Pro/Gly/Glu	Asn	Ile	Val	Val/Ile	
Glu/Val							
Leu	Thr	Ile	His	Val	Gln	Ser/Asn	Ala
Lys	Phe	Ala	Gly	Met	His/Met	Glu/Thr	
Gly/Asp							
Arg	Arg	Ser	Leu/Pro	Glu	Glu/Ser/Thr	Leu	
Ser/Lys/Phe							
Pro/Met	Ser/Gly		Lys/Tyr/Arg				
Ala/Phe/Gln							

Figure 28B

MMP-10 Stromelysin 2

P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
Arg	Ala	Ile	His	Ile	Gln	Ala	Glu
Gly	Pro	His	Leu	Leu	Val	Glu	Ala

Others

MMP-7 Matrilysin

P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
Ile	Pro	Leu	Glu	Leu	Arg	Ala	Gln
Gly	Leu	Gln	Met/Ala	Ile	Met	Val/Arg/Met	
Pro		Val	Pro/Gln	Met	Gln	Gly	
			Gly				

Figure 28C